Studies on the interaction between the entomopathogenic fungus, Metarhizium spp., and the desert locust Schistocerca gregaria (Forsk)

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Award date:
1995

Awarding institution:
University of Bath

STUDIES ON THE INTERACTION BETWEEN THE ENTOMOPATHOGENIC FUNGUS, METARHIZIUM spp., AND THE DESERT LOCUST SCHISTOCERCA GREGARIA (FORSK).

Submitted by Jeremy Paul Gillespie

for the degree of Ph. D.

of the University of Bath

1995.

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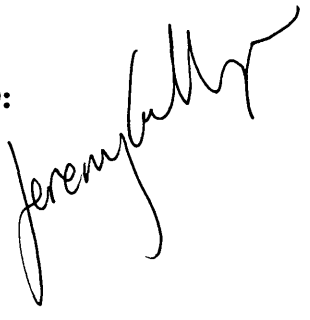

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"Nature never deceives us: it is always we who deceive ourselves"

Jean Jaques Rousseau

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Dedicated with love to Karen, and to
my parents, Colin and Janet

ACKNOWLEDGEMENTS

I would like to thank Dr. A.K. Charnley and Prof. S.E. Reynolds for their help, advice and encouragement during the course of this study. Thanks go to ChrisVennard for supplying me with locusts for the duration of this study. Thanks are also due to Dr. J.P. Aston for his help during the ELISA work and to Dr. C. Prior and all at the International Institute for Biological Control (IIBC) for their advice and donations of locusts.

I would also like to thank my friends in the School of Biology, not least of all Grainne, Ben and Justine for their support and advice. Thanks are also due to everyone who helped me get this finished. Thanks also to Dr. R.I. Samuels for his advice on the inhibitor work .

This project was funded by the SERC with CASE award from IIBC, UK.

Abstract.

This study investigates the relationship between the desert locust, *Schistocerca gregaria* and fungal pathogens of the genus *Metarhizium.* The main aims of the project were first to establish the immune reactions of *S. gregaria* in response to topical infection with *Metarhizium* spp. and second to investigate the ability of a chymoelastase (PR1) from a number of *Metarhizium* spp. to degrade different cuticle types and the involvement of the enzymes in isolate virulence.

S. gregaria responded to the application of conidiospores by mounting a cellular response. The total haemocyte count (THC) increased substantially after inoculation but declined to lower levels than controls. With the THC, individual sub-populations of cells changed in proportion to one another during infection. The proportion of plasmatocytes and granular cells declined during infection whilst the proportion of coagulocytes increased. In addition, the number of cellular nodules and the activity of phenoloxidase, and acid phosphatase increased and subsequently declined.

Initial changes in the immune system occurred within the first 2 days post-inoculation. Fungal hyphal bodies were first visible in the haemolymph 3 days after inoculation. Subsequently, the fungus proliferated rapidly and the host's immune system appeared to be compromised as all parameters investigated were lower than corresponding controls. If locusts were inoculated with high doses of *M. flavoviride*, fungal protoplast-like bodies were observed in the haemolymph 2 days after infection. Protein changes in the blood were also investigated. Whilst several proteins decreased in quantity during infection, new proteins appeared 3 days after infection. The origins of these proteins is discussed.

An inhibitor of the chymoelastase, PR1, has been identified in the plasma of *S. gregaria* and partially purified. A PR1 inhibitor from the plasma of *Manduca sexta* has been purified to homogeneity. This protein

has a molecular weight of 18.2 kDa and an isoelectric point of 4.6. It is unlike any of the protease inhibitors identified previously in *M. sexta* and does not cross-react with anti-serpin antibodies.

The role of PR1 in virulence has been investigated. The production of PR1 and PR2 by 19 strains of *Metarhizium* spp. was studied. There was no correlation between the quantity of enzyme(s) produced and the median lethal time of the isolates. The PR1 from each strain was partially purified and used to investigate the ability of the enzyme to degrade cuticle from different locations on *S. gregaria* as well as pupal cuticle from *M. sexta.* There was no correlation between the ability of the enzymes to degrade the types of cuticle and the median lethal time. However, the enzymes hydrolysed pharate abdominal cuticle better than adult abdominal cuticle or adult wing cuticle. The relationship between the properties of individual enzymes, the physicochemical characteristics of the cuticle from different regions and proteolytic activity is discussed. An Enzyme Linked Immunosorbant Assay (ELISA) for PR1 was developed. PR1 was not detected in the blood of either *M. sexta* or *S. gregaria* that had been topically infected with *M. anisopliae* (isolate ME1) using either the ELISA, a sensitive fluorimetric substrate or polyacrylamide gel electrophoresis.

Chapter 1

Literature Review

1.1 The biological control of insect pests.

Biological control can be defined as the "utilisation of natural or modified organisms, genes or gene products to reduce the effects of pests and diseases" (Cook, 1988). This definition of biological control encompasses the microbial control of insect pests since many micro-organisms are naturally occurring entomopathogens. Some researchers have tried to define such biologically based pest management programs as the utilisation of sterilised males, pheromones and pest resistant plants as biological control (Rosen, 1985). This only makes for confusion and here the term 'biological control' will be confined to natural organisms that associate with insects.

There is a continuing demand for more innovative and selective ways to control insect pests (Payne, 1988; Khachatourians, 1991). This has been necessitated by the deleterious effects of pesticides on the environment, increased reports of insecticide resistance and ever increasing costs of insecticide production (Charnley, 1989; Khachatourians, 1986). Disease causing organisms including viruses, bacteria, protozoa, nematodes and fungi have particular promise. This area of research is currently blossoming with a goal to establish microbial agents as environmentally safe, cheap to produce and efficient forms of pest control.

Approximately 1600 entomopathogenic virus isolates have been identified and classified into six groups based on their morphological appearance, nucleic acid type and pathology (Evans and Entwhistle, 1987). These are: (1) Baculoviridae (e.g. nuclearpolyhedrosis virus), (2) Reoviridae (e.g. cytoplasmic polyhedrosis virus), (3) Poxviridae (e.g. entomopoxvirus), (4) Iridoviridae (e.g. turquoise iridescent mosquito virus), (5) Parvoviridae (e.g.

densovirus) and (6) Picornaviridae (e.g. cricket paralysis virus). Of these, only the baculoviruses have been considered as microbial control agents because they do not share any biochemical similarities with viruses of vertebrates, plants or micro-organisms. At present a major disadvantage with the use of viruses as commercial bioinsecticides is that production and purification of the viral preparation is time consuming and labour intensive, resulting in high production costs (Payne, 1988).

Approximately 100 entomopathogenic species of bacteria have been identified (Krieg, 1987). These have been classified into the following four groups based on their pathogenicity to insects: (1) obligate pathogens (e.g. *Bacillus popillae*), (2) crystalliferous spore-forming bacilli (e.g. *B. thuringiensis*), (3) facultative pathogens (e.g. *B. sphaericus*) and (4) potential pathogens (e.g. *Pseudomonas aeruginosa*) (Khachatourians, 1986). Most entomopathogenic bacteria, belonging to the *Clostridium, Pseudomonas* and *Serratia* groups have failed as biocontrol agents due to inconsistent virulence, difficulty of *in vitro* production or safety (Luthy, 1986). Only certain *Bacillus* spp. have shown any potential as insect specific pest control agents (Luthy, 1986). Commercial production of bacterial entomopathogens, particularly *B. thuringiensis*, occurs world-wide and has been the major source of microbial insecticide to date (Luthy, 1986; Khachatourians, 1986).

There are many entomopathogenic protozoans which belong to the following four phyla: (1) Sarcomastigophora (e.g. *Malamoeba locustae*), (2) Ciliophora (e.g. *Tetrahymena* spp.) (3) Apicomplexia (e.g. *Mathesia gradris*) and (4) Microspora (e.g. *Nosema* spp.; Maddox, 1987). Protozoan infection is chronic and benign, often causing reduced host development rates and fecundity. Due to this, they have not been successfully used in the environment as fast acting microbial insecticides and it is unlikely that they will be in the future (Maddox, 1986; Payne, 1988). Furthermore, commercial production is

expensive, as the preparation of protozoans requires extraction from infected hosts followed by centrifugation.

The multicellular nematodes of the genera *Heterorhabditis* and *Steinernema* are usually described as "microbial biocontrol agents" because they are mutualistically associated with entomopathogenic bacteria of the genus *Xenorhabdus* (Poinar, 1986). These bacteria are exclusively held in the intestine of these two nematode genera. Most nematodes enter the host through the mouth or anus and invade the haemocoel by penetrating the gut wall. Furthermore, some *Heterorhabditis* spp. may gain access to the haemocoel by penetration of the host cuticle. This relationship is symbiotic as the nematodes feed on the bacteria and decaying host tissue (Wouts, 1984) whilst the bacteria live on the host insect. Nematodes present an interesting case as biocontrol agents due to their wide host range and quick speed in killing insects. Moreover, there is no evidence to suggest any natural or acquired resistance to *Xenorhabdus* spp. (Poinar, 1986). However, disadvantages for their use include problems with storage and shipping and the occurrence of natural enemies (Poinar, 1986). Currently, they represent a commercially available biological product for use against insect pests in the soil (Payne, 1988).

More than 700 species of fungi from approximately 90 genera are pathogenic to insects (Charnley, 1989). These fungi vary widely in ease of mass production, host range and ability to initiate epizootics. Therefore, they provide a wide spectrum of organisms with potential for development as pest control agents (Roberts and Humber, 1984). Fungal insect pest control agents are specific, harmless to beneficial organisms and can persist to exert long-term control. Whilst bacteria and viruses are also proficient in these areas, they require access to the gastrointestinal tract of the host for disease to result. Although some entomopathogenic fungi enter the gut or respiratory tract, the majority invade their host through the external cuticle. This is a unique mode of entry for a micro-organism and characteristic of the fungi. This gives the fungi

great potential for the control of, for example, the non-feeding stages of many insects or Hemipterans which have sucking mouth parts and do not ingest pathogens. However, the exploitation of the fungi as a biocontrol agent has been slow in progressing. Despite the great number of species identified, only about 20 have been studied intensively as potential biological control agents (Zimmerman, 1986). The major reason appears to be that they are not seen as having major potential by commercial companies. The host-specificity, slow speed of action and the common belief that mycoinsecticides are not effective insect control agents (Payne, 1988) have contributed in the slow development. An understanding of the mechanisms of fungal pathogenesis and the basis of host defence will aid in the development of faster killing, more effective mycoinsecticides.

1.1.2. Locusts and grasshoppers as targets for biological control.

Since the beginnings of civilisation, grasshoppers and locusts have been some of the great agricultural pests, having the capacity to form large conspicuous congregations. Grasshoppers and locusts are close biological relatives belonging to the same insect family: the Acrididae. Locusts may be differentiated from grasshoppers by having a distinctive ability to change their morphology and behaviour when they aggregate in large numbers (Rowley, 1993). In terms of successful adaptation to extreme conditions such as the Sahel in Africa, the important factors for grasshopper and locusts are their capacity to reproduce fast, the flexible organisation of their life cycle and their ability to undergo "gregarisation" and migrate. This change is accompanied by increasing mobility and activity resulting in a dense population consisting of bands (groups of wingless nymphal instars) and swarms (groups of adult locusts). An average swarm contains 20-50,000,000 insects per sq. km. This number of

insects is capable of eating 40-100 tons of vegetation per day. Swarms of these insects cause enormous damage to world crops (Brader, 1982). For example, in the summer of 1985, monetary losses due to grasshopper infestations on the Canadian prairies alone was estimated at $100,000,000 (Bidochka and Khachatourians, 1991)

Information on the chemical control of these insects is well documented (see Steedman, 1990) with the organochlorine, dieldrin, being one of the most effective insecticides used in the past to control locust outbreaks. Due to environmental concerns and risks to humans and livestock, biological control measures have been researched into as alternatives to chemical pesticides. At present, the large scale application of broad spectrum insecticides is the only effective control measure for both locusts and grasshoppers. However, the unsatisfactory control achieved by chemical products during the 1986-1989 plague led to renewed interest in the development of alternative biological based measures to control locusts and grasshoppers.

Grasshoppers and locusts are a host to a wide range of pathogenic micro-organisms including fungi, protozoa, viruses and rickettsia (Henry *et al.*, 1985; Streett and McGuire, 1990). Many entomopathogenic micro-organisms have now been cultured and can be maintained *in vitro* in the laboratory and be produced on an industrial scale (Khachatourians, 1991). Most biological control measures are host specific and thus less environmentally harmful. A range of biological control agents that could be used against locusts and grasshoppers has been reviewed elsewhere (Prior and Greathead, 1989; Bidochka and Khachatourians, 1991).

The bacterial pathogen, *Bacillus thuringiensis*, has been employed with great success to control Lepidopteran, Dipteran and Coleopteran pests. However, no Orthopteran active strain of *B. thuringiensis* has been found. Opportunistic pathogens such as *Serratia marcescens* and

Pseudomonas aeruginosa can infect locusts but are also potentially harmful to mammals.

A variety of entomopoxviruses (EPVs) are pathogenic to acridids but are not considered as viable control agents because they lack host specificity. The protozoans, *Malamoeba locustae* and *Nosema* spp. have been investigated for Acridid control. Of these, *Nosema locustae* is used to control grasshoppers in North America. It causes a debilitating disease which reduces the life-span and fecundity of its host (Brooks, 1990). The problem with protozoans as control agents lies in the fact that the disease is chronic rather than acute and does not bring about a quick removal of the pest

Several fungal species are pathogenic to locusts and grasshoppers. These include *Entomophaga grylli*, *Metarhizium* spp, *Beauveria* spp, *Paecilomyces* spp. and *Verticillium* spp. (Prior and Greathead, 1989; Bidochka and Khachatourians, 1991). The fungi have great potential as effective biological agents as they are cheap to produce, host specific, applicable using conventional methods and act rapidly. More importantly, these fungi establish a mycosis after penetration of the host cuticle. This is unlike the other microbes described above which have to be ingested.

The formulation of the entomopathogenic fungi *M. anisopliae* and *M. flavoviride* for biological control has been perceived as a viable non-chemical treatment of locusts. Indeed, *M. flavoviride* is being developed as a potential control agent for the efficient control of *Schistocerca gregaria* and other locusts and grasshoppers in West Africa (Prior and Greathead, 1989).

It is clear that the entomopathogenic fungi, particularly *M. flavoviride*, have great potential as biological control agents, whether applied alone or in tandem with a low dose of insecticide.

1.2 The cells and tissues of the insect immune system

The main components of the insect immune system are the blood cells (haemocytes) together with other cells and tissues such as the cuticle, fixed haemopoietic tissues, nephrocytes/pericardial cells and the fat body (Table 1). The following description covers the structure and significance of these cells/tissues in the cellular and humoral immune reactions of the host.

1.2.1 The cuticle and alimentary canal

Both the cuticle and alimentary canal are important barriers against infection as they are the site of pathogen/parasite entry into the haemocoel. The cuticle consists of an insoluble, multilayered matrix composed of three main components; protein, lipid and chitin. The outermost layer comprises of a cement layer, a wax layer, the outer and inner epicuticle. The outer epicuticular layer is made up of quinone-linked proteins whilst the inner epicuticle is lipoprotein stabilised by quinones. The whole epicuticle is less than 1 μm in thickness. Beneath this is the procuticle (10-50 μm thick) which has been sub-divided into exo-, meso- and endo-cuticle on a histochemical basis. The procuticle is made up of chitin fibres embedded in a protein matrix (Kramer and Koga, 1986). Chitin is a linear polymer of ß-1,4-linked N-acetyl D-glucosamine units and exists in the form of a glycoprotein or chitin-protein complex (Kramer and Koga, 1986). The nature of the proteins associated with chitin and the type of links between proteins give the cuticle its characteristic hardness (Jeaunieux, 1984). A highly sclerotised cuticle will have lots of protein cross-linked to one another by quinones. The cuticle has been considered to be a mechanical barrier that effectively safeguards against most pathogenic micro-organisms (Charnley, 1990; St. Leger, 1991).

Table 1. The cells and tissues of the insect immune system.

Cells/Tissue	Function
Cuticle/Alimetary Canal	Physicochemical barrier to invasion; mechanical prevention; microbicidal components (?)
Free Blood Cells (haemocytes)	Recognition of foreignness; plasma gelation; wound healing; nodule formation/encapsulation; production of lysozyme, agglutinins, pPO and other humoral factors
Fixed cells in haemopoietic tissue	Phagocytosis; synthesis of antimicrobial factors
Pericardial Cells	Lysozyme synthesis; production of other antibacterial factors (?); sequestration of soluble antigenic material
Fat Body Cells	Synthesis of induced immune proteins such as cecropins and lectins

The fore- and hind-gut of most insects is protected from possible infection by a cuticle lining which is absent in the midgut region. However, at this site, the epidermal cells are covered by a proteinaceous sheet called the peritrophic membrane (PM; Chapman, 1985). The PM consists of a network of chitin fibrils set in a carbohydrate protein matrix (Chapman, 1985). The functions of the PM are not known conclusively but it is thought that one function is to serve as a mechanical barrier to infection by microbial agents (Orihel, 1975).

1.2.2 Haemocytes

Haemocytes are cells which are freely circulating or sessile and attached to various tissues throughout the host body cavity. The number and nomenclature of insect haemocytes has been a matter of considerable controversy for many years. The problems in the classification of insect blood cells are due mainly to the sheer diversity in insect forms which is reflected in the great variation in the structure of the blood cells present (Ratcliffe, 1993). Gupta (1985), based on ultrastructural observations in the literature, reports that there are seven major types of haemocyte common to many species of insect with a few specialised cells peculiar to specific species. However, other authors such as Price and Ratcliffe (1974) and Zachary and Brehelin (1986) contend that there are different numbers of haemocyte types depending on species. Recently, cell types have been identified by the nature of their cell surface determinants as detected by staining with lecithin and monoclonal antibodies (Mullett *et al.*, 1993a). However, the problem in obtaining a unified classification of blood cells with this method is that the antibodies may well be genus specific. I shall utilise the classification of Gupta (1985) for the discussion of haemocyte types below. This classification scheme allows for 7 haemocyte types: prohaemocytes; plasmatocytes; granular cells; coagulocytes;

spherulocytes; oenocytoids; and adipohaemocytes; and is found in most general entomology textbook.

Prohaemocytes (PRs) are small spherical cells that are typically 6-13 μm in diameter. There is a large nucleus present with a narrow rim of homogenous cytoplasm around it. The cytoplasm exhibits several features of undifferentiated cells: numerous free ribosomes; small quantity of rough endoplasmic reticulum (RER); and few mitochondria. PRs may be numerous, rare or absent, depending upon the developmental and physiological state of the insect at the time of observation. It is generally thought that PRs are stem cells that transform into other haemocytes (Jones, 1962; Gupta, 1979).

Plasmatocytes (PLs) are highly polymorphic cells and are round or spindle shaped with variable size (10-45 μm). After bleeding they become adhesive and amoeboid (Ratcliffe and Rowley, 1987). The cytoplasm is highly differentiated and can contain few to many phase-dark granules (Rowley and Ratcliffe, 1981). While the presence of granules has led to confusion of the identification of this cell type, the amoeboid movement and spreading capacity of the cell makes identification easier. The PLs are actively phagocytic and the many lysosomes found within the cytoplasm contain an array of antimicrobial agents such as hydrolytic enzymes and lysozyme (Chain and Anderson, 1983a). PLs are also found near the centre of capsules and nodules that form around foreign bodies (Gotz, 1986).

The granular cells (GRs) are round or oval, 8-20 μm in diameter and readily identified in many insect species (Price and Ratcliffe, 1974). They contain a large number of granules accompanied by well developed Golgi bodies. The cells are unstable *in vitro* and will rapidly degranulate. Degranulation has been implicated in prophenoloxidase (pPO) activation (Schmit *et al.*, 1977), plasma coagulation (Rowley, 1977) and possibly acting as opsonins (Ratcliffe and Rowley, 1979; Ratcliffe *et al.*, 1984). The GRs are the first cells to participate in encapsulation and nodule formation.

Coagulocytes (COs) are round to oval cells (3-30 μm) which are involved in haemolymph coagulation. The COs have been mistakenly identified as GRs in several insect species (Gupta, 1985). This stems from the fact that the COs contain granules and are highly unstable *in vitro* and rapidly degranulate. However, they can be distinguished from GRs because they are weakly periodic acid-Schiff positive (Costin, 1975). It is not clear whether all the GRs possess the ability to function as clotting cells or whether there is a subpopulation of specialised fragile cells (Ravindranath, 1980; Bohn, 1986). Indeed, it has been suggested that, in species that have both COs and GRs, the former play more of a role in haemolymph coagulation (Rowley, 1977).

Spherulocytes (SPs) are round or ovoid cells with variable size (10-25 μm). They have a small nucleus with a cytoplasm containing spherules that are characteristic of this type of cell. The number of spherules may vary from few to very many and have been reported to contain neutral or acid mucopolysaccharide and glycomucoproteins (Gupta and Sutherland, 1967; Ratcliffe, 1975; Cook *et al.*, 1985). However, their role in defence still remains unknown.

Oenocytoids (OEs) are round to oval shaped cells and are easily identified by their large size (54 μm or more) and homogenous cytoplasm. They are filled with many large mitochondria whereas the ER and Golgi apparatus are poorly developed. Crossley (1975) found that after incubation of cells with tyrosine, dihydroxyphenylalanine (DOPA) or dopamine the cells turned black indicative of PO activity. Furthermore, Iwama and Ashida (1986) have presented immunochemical evidence of pPO being present in the OEs of *Bombyx mori*. Despite this, the function of OEs in defence is unknown.

Adipohaemocytes (ADs) are small to large spherical or oval cells with variable size (7-45 μm). The nucleus is small and the cytoplasm contains characteristic fat droplets of variable size, well developed Golgi bodies, mitochondria and polyribosomes. In addition, non-lipid vacuoles and granules

may also be present. Histochemically, the granules have been reported to contain a periodic acid-Schiff-positive substance. They have been reported to be phagocytic and it has been suggested that they are a form of granule cell (see Gillott, 1980).

In addition to these common haemocyte types, some insects contain special cells. Thrombocytoids are restricted to various Dipterans that do not contain coagulocytes (Zachary and Hoffmann, 1973). They are generally anucleate and capable of fragmenting and rapidly agglutinating and are highly phagocytic. They have been thought to be a specialised form of PL (Price and Ratcliffe, 1974) involved in wound healing (Zachary *et al.*, 1975; Kaaya and Ratcliffe, 1982). Furthermore, *Drosophila melanogaster* possess crystal cells (Rizki and Rizki, 1980). These cells enclose large prominent paracrystalline inclusions in the cytoplasm which are rich in pPO. Thus, they are presumed to be involved in the process of melanisation and implicated in immune responses.

1.2.3 Fixed cells in haemopoietic tissue

Apart from the transiently sessile haemocytes in many insects, other populations of permanently attached haemocytes are involved in insect cellular defences. These haemopoietic organs have been described in such insect species as *Galleria mellonella* and *Locusta migratoria* (Jones, 1970; Hoffmann *et al.*, 1974). They may be diffuse structures and little more than an accumulation of a few sessile haemocytes (e.g. in mosquito) or more highly organised and surrounded by a limiting membrane (e.g. in many Orthopterans; Hoffmann *et al.*, 1979). Hoffmann *et al.* (1974) showed that the haemopoietic organs in the insect body are capable of responding to invading parasites/pathogens. For example, the cells lining the gut of *Amphimallon majalis*, injected with *Bacillus popillae*, endocytose and destroy the bacteria

(Kawanishi *et al.*, 1978). Brehelin and Hoffmann (1980) showed that a specific cell type, the reticular cell, is peculiar to haemopoietic organs. This cell does not enter the circulation but is highly phagocytic and is responsible for the production of proteinaceous antibacterial material liberated into the blood. The overall importance of haemopoietic organs in comparison to free haemocytes in the clearance of foreign material from circulation is unknown and may vary from one species to another.

1.2.4. Nephrocytes/Pericardial cells.

These cells are closely organised with the heart and alary fibres. they are often extensively infiltrated into the fat body tissue and are found in many insect species (Crossley, 1972). Crossley (1972; 1983) has found that they pinocytose small particulate matter such as dyes, colloidal carbon and tracer molecules such as ferritin. Ultrastructural studies of these cells showed that the peripheral cytoplasm contained a large number of pinocytotic vesicles with a plasma membrane rich in deep infoldings (Crossley, 1972). Based on these ultrastructural findings, it has been suggested that the pericardial cells/nephrocytes are involved in the detoxification and elimination of soluble material formed after the dissolution of microbial/macrobial agents. These cells have been likened to the reticuloendothelial system of vertebrates (Wigglesworth, 1970), a system known to remove foreign materials and denatured proteins from blood. Pericardial cells have also been shown to produce lysozyme in *Calliphora erythrocephala* and *Manduca sexta* (Crossley, 1972; Russell and Dunn, 1990). Russell and Dunn (1990) have suggested that the fat body is the primary source of the enzyme and that pericardial lysozyme may function in the intracellular lysosomal degradation of pinocytosed fragments of bacterial invaders.

1.2.5 Fat body

In insects, the fat body has been regarded to play much the same role as the liver does in mammals. It functions as the site of synthesis and storage of lipids, proteins and carbohydrates. The fat body consists of irregular masses of rounded or polyhedral cells which are in intimate contact with the haemolymph and haemocytes. Faye and Wyatt (1980) have shown that the fat body removed from immunised cecropia moths, *Hyalophora cecropia*, is capable of synthesising a range of antibacterial proteins (see later). This also occurs in larvae of *G. mellonella* and *M. sexta* (DeVerno *et al.*, 1984; Dunn *et al.*, 1985). Azambuja and Garcia (1987) have suggested that lysozyme is released from the fat body after infection of *Rhodnius prolixus.* A variety of proteins have been purified from the fat bodies of several insects indicating that the organ is a principal factor in the production of humoral immune factors. Furthermore, the fat body has been shown to be the site for the production of agglutinins (Kubo *et al.*, 1984).

1.3 Arthropod immune responses

In some parts of the literature, the term 'immunity' is confined to phenomena involving lymphocytes or immunoglobulins This is unfortunate because the term 'immunity' here is justified in that the definition of the word in the Oxford English Dictionary is given as the ability of an organism to resist or protect against disease. In contrast to vertebrates, arthropod immunity is not based on the presence of immunoglobulins and interactive lymphocyte subpopulations. An alternative word for immunity would be to use 'defence reactions' which is ambiguous as it could be taken to mean reactions employed by insects to defend themselves from other insects or predators, e.g. the sting of a bee or a wasp is a defence reaction. With an open circulatory system,

invertebrates must have a rapid and immediate non-inducible defence and coagulation mechanism to entrap parasites and prevent blood loss after wounding.

The arthropod immune defence system includes a range of cellular and humoral components which are summarised in Table 2 and discussed in more detail below.

1.3.1 Cellular immune responses

Many functions have been assigned to haemocytes including macromolecular transport (Chino, 1985), haemolymph coagulation (Bohn, 1986) wound healing (Gunnarsson, 1987), phagocytosis and encapsulation (see Gotz and Boman, 1985). The involvement of haemocytes in coagulation, wound healing and immunity ensures an efficient means of internal defence. The mechanisms of the responses to infection and injury are described in this section.

1.3.1.1 Clotting/coagulation and wound healing

The route of entry for many opportunistic pathogens into the haemocoel is through wounds in the cuticle. Parasites such as entomopathogenic fungi and parasitic wasps directly pierce the cuticle creating wounds. The host must close these wounds quickly and efficiently in order to prevent excess body fluid loss, impaired function and infection. In arthropods, the injury is rapidly sealed by extrusion of the fat body or gut, by haemolymph coagulation and melanin deposition, or by muscular constriction (Gregoire, 1970). Haemocytes then migrate to the wound site which may be mediated by the production of lymphokine-like factors secreted by the epidermis (Cherbas, 1973; Gunnarsson, 1987). Once there, they aggregate plugging the wound and phagocytose or

Table 2. Summary of processes involved in host defences and the factors involved in them (after Rowley and Ratcliffe, 1987).

Cellular	Humoral
Wound healing (haemocytes and epidermal cells)	Wound healing (lymphokine-like substances)
Coagulation (COs, haemocyte-derived coagulogen; pPO system involvement)	Coagulation (plasma-derived coagulogen
Phagocytosis (PLs)	Phagocytosis (opsonic involvement by pPO cascade components)
Encapsulation-type responses (multi-cellular)	Encapsulation (melanisation-pPO)
pPO system synthesis (proenzyme, proteases by OEs?)	pPO activation (non-self recognition; humoral factors e.g. β-1,3-glucans)
Agglutinins (synthesised by fat body; receptors on haemocytes?)	Agglutinin activity (recognition)
Lysozyme synthesis (fat body, pericardial cells)	Lysozyme activity, lysozyme co-factors
Lysosomal enzymes (PLs)	Naturally occurring antimicrobial factors
	Inducible, partially non-specific antibacterial proteins (e.g. cecropin; hemolin)

encapsulate cell debris and potential invaders. Wound closure is facilitated either with or without gelation of the blood (Ratcliffe *et al.*, 1985). Numerous haemocytes migrate into the area and close the wound by cell aggregation. Once at the wound site, the cells rapidly discharge their contents and the plasma gelates to strengthen the haemocyte plug.

In insects, the coagulation process involves a cascade reaction (Bohn, 1986). Bohn has performed a great deal of work in the area of haemolymph clotting using *Leucophaea maderae* as a model system. The author has found that the insect has two clotting proteins, the haemocyte coagulogen which is contained in the granules of the coagulocytes and released during rupture of the cells and the plasma coagulogen which has been identified by Chino *et al.* (1981) as lipophorin. Clotting is initiated with the rupture of the coagulocytes provided that calcium cations are present (Bohn and Barwig, 1984). The ruptured haemocytes release the haemocyte coagulogen and other substances into the surrounding haemolymph. The haemocyte- and plasma-derived coagulogens associate and become cross-linked to form the haemolymph clot. Cross-linking requires the presence of calcium ions and the activity of a haemocyte-derived sulphide factor (Bohn and Barwig, 1984). In the absence of plasma coagulogen, the haemocyte coagulogen can also form a coagulum called the haemocyte gel. This gel soon ages and loses the ability to cross-link with the plasma coagulogen. Although the coagulation process has been elucidated in *L. maderae*, it remains unclear as to whether this process can be generalised to accommodate all insects (Bohn, 1986). However, it would appear that there is some conservation between species as Bohn (1986) reports that plasma coagulogen from *Periplaneta americana* can make full clots with the haemocytes of *L. maderae*. However, in many Dipterans gelation is rarely found and, instead, small cytoplasmic fragments, thrombocytoids, aggregate and prevent haemolymph loss (Zachary and Hoffmann, 1973).

Gunnarsson (1987) has described haemocyte behaviour following wounding of the cuticle. If wounding was penetrative, then no cells were found on the basement membrane immediately around the wound site. However, superficial wounding which did not disturb the epidermis resulted in haemocyte movement to the basal membrane resulting in an aggregate below the injury point. Those cells on the periphery of the aggregate were generally rounded with the appearance of circulatory haemocytes. In the case of the scarified injury, the first haemocytes to arrive spread out on the basement membrane and the other haemocytes adhered around and upon them producing a multilayered structure resembling a capsule (see later). These cells were observed to be granular and agranular and remain unidentified. The final phase of the reaction consisted of melanisation, starting at the inner surface of the endocuticle and developing inwards into the aggregate of haemocytes.

In light of these findings, Gunnarsson (1987) suggested that, because scarification of the cuticle and penetration by hyphae from the entomopathogenic fungus *M. anisopliae*, 12 h after topical application, did not involve penetration of the epidermis, then a stimulus inducing the haemocytes to adhere to the basement membrane must pass through the epidermis and the basement membrane in order to recruit haemocytes to the site. Nyhlen and Unestam (1980) have observed similar events in crayfish but only if the wound is completely covered with water. The authors suggest that osmotic changes within the integument could be responsible for the induction of a wound healing reaction. This is unlikely to be the case in insects as osmotic changes had no effect on locust coagulation (Gunnarsson,1987).

1.3.1.2 Phagocytosis

Small biotic particles such as bacteria and yeast as well as abiotic particles such as latex beads and colloidal carbon are internalised by circulating

PLs. Ratcliffe and Walters (1983) have shown that low doses of bacteria (less than $10^3/\mu l$) induce removal by phagocytosis. Doses above this threshold level resulted in nodule formation.

The proportion of the haemocyte population that is phagocytic depends upon the dose of the particle (Ratcliffe and Walters, 1983), the time post-injection at which the haemolymph is examined and the prior exposure of the insect. Mohrig *et al.* (1979) found that prior injection of *G. mellonella* with latex beads increased the ability of the haemocytes to ingest a subsequent dose of *B. thuringiensis subtoxicus*. Phagocytosis can also be non-specifically stimulated by the injection of saline into *S. gregaria* (Gunnarsson, 1988a) or sepharose beads into *P. americana* (Dularay and Lackie, 1985). Wiesner and Gotz (1993) have shown that monolayers of PLs isolated from *G. mellonella* have 64% phagocytic activity against sterile silica beads.

Smith and Soderhall (1986) have shown that crustacean hyaline haemocytes can become more phagocytically competent after the addition of ß-1,3-glucans or lipopolysaccharide (LPS) *in vitro*. However, Soderhall *et al.* (1986) subsequently pointed out that this phenomenon arose because the microbial products initiated degranulation of the granular and semigranular Haemocytes which, in turn, liberated the components of the pPO cascade. These components would then become activated and act as opsonins to direct phagocytosis. Gunnarsson (1988a) has shown this phenomenon to occur in insects as well. The author observed that ß-1,3-glucans increase the proportion of phagocytically competent haemocytes in *S. gregaria in vivo*. Furthermore, Mullett *et al.* (1993b) showed that PL from *G. mellonella* could increase the phagocytosis of *B. cereus* in the presence of laminarin.

There have been a number of histological and ultrastructural examinations of phagocytosis by haemocytes and results have indicated that the mechanism is essentially similar to that seen in other types of phagocytic cell such as mammals (Rowley and Ratcliffe, 1979). Mammalian leukocytes

possess a wide range of intra- and extra-cellular killing mechanisms (Horowitz, 1982). These include; the oxygen dependant killing (myeloperoxidase-hydrogen peroxide-halide system) of neutrophils and activated macrophages; oxygen independent mechanisms such as the proteinases, esterases, acid phosphatases and lysozyme found in lysosomes and secreted into the phagosomes and extracellular fluid; and molecules such as major basic protein and cationic protein secreted by the eosinophils and responsible for killing parasites (Mims, 1988). Phagocytosis in normal human polymorphonuclear leukocytes is accompanied by a burst of metabolic activity (Horowitz, 1982). The most marked changes are a stimulation of respiration and an increase in the amount of glucose oxidised via the hexose monophosphate shunt. However, these reactions are not triggered during phagocytosis by *Blaberus craniifer* cells (Anderson, 1974). Furthermore, Anderson *et al.* (1973) have reported that haemocytes of *B. craniifer* do not contain myeloperoxidase whilst Molyneux *et al.* (1986) have found no evidence for the presence of hydrogen peroxidase in *S. gregaria* cells.

Despite the fact that the presence of lysosomal marker enzymes has been shown in several insects (Rowley and Ratcliffe, 1979), we remain ignorant of the mechanisms involved in the killing of micro-organisms. However, one marker enzyme, acid phosphatase, has been shown to be involved in the immune responses in *M. sanguinipes* (Vincent *et al.*, 1993).

1.3.1.3 Cellular Encapsulation

Phagocytosis predominates when the haemocoel is exposed to small numbers of foreign particles. However, encapsulation and nodule formation are the common types of immune reaction in arthropods against larger pathogens and parasites, introduced foreign bodies or injected particulate materials. Cellular encapsulation consists of the formation of a capsule-like envelope

around foreign objects. This capsule is formed by attaching blood cells. Cell-mediated encapsulation occurs in 2 forms: a) nodule formation against small particulate matter and (b) capsule formation which occurs if the foreign object reaches or exceeds the size of the blood cells (Gotz, 1986).

Cellular encapsulation has been observed in all insect groups investigated (Ratcliffe, 1986). Live organisms such as bacteria, fungi, nematodes, and eggs or larvae of parasitic insects such as braconids or tachinids are common elicitors of such a reaction (Ratcliffe *et al.*, 1985). Inert substrates such as latex or sepharose beads, araldite or cotton threads have also been shown to induce the formation of a cellular capsule (see Gotz and Boman, 1985). The reaction against living organisms is strong and always accompanied by melanisation whilst most inert particles only induce weak reactions resulting in nodules with little or no melanisation.

The cellular capsule comprises of 3 layers (Dunn, 1986; Gotz, 1986; Ratcliffe and Rowley, 1987). The centre of the capsule or nodule consists of the provocating agent together with disintegrated GRs. The middle layer consists of extremely flattened PLs with an outer layer of normally shaped PLs in loose connection.

In order to encapsulate a foreign object, contact must be initially established (Gotz, 1986). For this to occur, it is presumed that random contact of the haemocytes with the objects takes place. This seems likely due to the turbulence of the blood stream and the narrow passages of the circulatory system (Miller, 1985).

The first blood cells reacting to non-self matter are generally accepted to be the GRs (Gagen and Ratcliffe,1976; Schmit and Ratcliffe, 1977; 1978). However some authors have reported that PLs are the first cells recruited to encapsulation (Poinar *et al.*, 1968). In the following account, cellular encapsulation is based on the assumption of Gotz (1986) that GRs are the first cells to react with foreign bodies.

In order for activation of blood cells to occur, reactions will occur at the cell membrane. Very little is known about the molecular nature of recognition in encapsulation reactions. However, cell-bound receptor molecules, cell-bound agglutinins, components of the phenoloxidase cascade as well as differences in the electrical charge of the cell membrane and the foreign object have all been implicated as factors of recognition (Gotz and Boman, 1985; Soderhall, 1982; Takle and Lackie, 1985).

Coagulocyte cells may play a role in the recognition of foreigness as these cells contain factors associated with the pPO system and agglutinins. Thus, CO degranulation in response to invading micro-organisms will release these components (Ratcliffe, 1993). A fibrillar substructure within the vacuoles becomes visible during this process. After degranulation, the once electron-dense granules are observed to be light (Gotz, 1986) with the electron dense material observed surrounding the discharging cell. If a foreign object is present in the neighbourhood of the discharging GRs, then the material adheres to the foreign surfaces. More often than not, the GRs completely disintegrate liberating a pot-pourri of compounds such as coagulogens, agglutinins, lysozyme, 'sticky' proteins (pPO system components) as well as chemotactic factors which attract PLs (Davies *et al.,* 1988). Brookman *et al.* (1989a) found that the capsular polysaccharide from *Klebsiella pneumoniae* significantly enhanced nodule formation by haemocytes of *L. migratoria* and *S. gregaria.* They also found that the lysozyme soluble fraction of peptidoglycan activated nodule formation. Furthermore, Brookman *et al.* (1989b) found results were similar to those obtained for pPO activation with the same samples.

It seems likely that agglutinins play the most important functional role in recognition. However, the question of how they interact with haemocytic surfaces to form bridging molecules attaching the foreign particles to the blood cells is unknown. It is possible that exposure of endogenous lectins to foreign molecules may induce modifications in the lectin so that it can interact with

carbohydrate molecules on the haemocytic surface (Miranpuri and Khachatourians, 1993).

Phenoloxidase has been extensively implicated in the recognition of non-self molecules (Soderhall, 1982; Rowley *et al.*, 1986). Prophenoloxidase has also been implicated in the recognition of foreigness (Ratcliffe *et al.*, 1984). Haemocyte monolayers from *G. mellonella, L. maderae* and *B. craniifer* were overlaid with β-1,3-glucans and lipopolysaccharide (LPS) which are activators of pPO in these insects. The effect was to enhance phagocytosis of test bacteria (Leonard *et al.*, 1985). This seemed to indicate that, since activation of pPO generates sticky proteins that adhere to foreign invaders (Soderhall *et al.*, 1979), then the test bacteria become coated in a sticky opsonic material that is recognised as being foreign by phagocytes. Unfortunately, this method proved unsuccessful in *S. gregaria* (Dularay and Lackie, 1985) in which sepharose beads were coated with PO-associated sticky proteins. If the pathogen/parasite has the appropriate stimulatory product (ß-1,3-glucan, lipopolysaccharide) on its surface, the GRs disintegrate onto the foreign surface. This discharge process corresponds to the activation of the pPO system and the transgressor becomes covered in sticky proteinaceous substances which may represent recognition markers for other cells (Rowley *et al.,* 1986). PO has been reported to be present in the haemocytes of the haemolymph (Leonard *et al.*, 1985a) although the exact localization to a subpopulation has only been reported once to date (Mead *et al.*, 1985). The contents of discharged GRs could conceivably represent activated PO or activators of PO which is known to be present in the haemolymph plasma (Saul and Sugumaran, 1987; Bidochka *et al.*, 1989), PO is almost certainly present at the interface region because melanization occurs first at the innermost surface of the nodule or capsule where the contents of the granular vacuoles are. Due to the sticky properties of the discharged material, clumps of GRs and particulate material are formed or, if larger foreign objects are involved, attachment of GRs to the foreign surface occurs. Marmaras *et al.*

(1993) suggested that certain cuticular proteins were responsible for non-self recognition in *Ceratitis capitata* because they were able to bind to the surface of *E. coli in vitro*. Subsequent to this, a reactive tyrosine derivative is generated which immobilises and probably kills the bacteria. These recent findings indicate that, instead of just being an inert preventative barrier, the insect cuticle is an active participant in host defences.

In both nodule and capsule formation, GRs attach to agglutinated foreign particles or to the surface of larger objects. The presence of activated GRs attracts PLs which in turn form an envelope of flattened blood cells. The presence of molecules for attachment to foreign bodies is crucial. This was illustrated by Mullet *et al.* (1993b) who showed that monoclonal antibodies generated against haemocytic surfaces can prevent the formation of a capsule and attachment of bacteria to granular haemocytes.

It is thought possible that discharged material from these GRs induces different types of blood cells to spread (see section 2.3.2.3). This would explain why various blood cells have been reported to participate in the formation of the cellular envelope (see Gotz, 1986).

The next step in the formation of a capsule is to establish a multicellular entity surrounding the foreign object. This is achieved when the PLs flatten above one another (Schmit and Ratcliffe, 1977). This flattening is accompanied by the formation of desmosomes, microtubules and microfilaments (Gotz, 1986) which may contribute to the mechanical strength of the envelopes. Once again, there seems to be some controversy surrounding the involvement of the PLs in capsule formation. Brehelin *et al.* (1975) believe that the GRs are involved in the formation of the middle part of the envelope in *L. migratoria* and *Melonontha melonontha.*

After the PLs have flattened, electron dense material appears in the intercellular spaces. Approximately 6 h after the onset of encapsulation, melanization of the nodule or capsule becomes apparent. The first site of

melanization is always the surface of the foreign object. This is the most positive evidence for a role of the components of the pPO cascade in the reaction. It is important to correlate the degree of encapsulation with the proportion of disintegrated haemocytes because melanization can continue for days, expanding continuously from the centre to the middle and outer layers of the capsule (Gotz and Boman, 1985). Complete melanization has only been observed in capsules surrounding parasites such as nematodes and parasitoids (Salt, 1963; Gotz and Boman, 1985). It is worth noting that melanization is not always a consequence of nodule formation whereas it is for encapsulation (Gunnarsson and Lackie, 1985; Guzo and Stoltz, 1987)

An important point in encapsulation is what factor(s) regulates its termination. Only the innermost blood cells have had any direct contact with the foreign body. Further haemocytes can only attach to bound blood cells. The signals that initiate their attachment could originate from those haemocytes already adhering. It is possible that as the capsule increases in thickness, the signal recruiting further cellular participants is dampened and ultimately ceases as the capsule reaches a finite limit (Gotz and Boman, 1985). This concept of a stimulus gradient is supported by the observation that the outer cells tend not to change shape. Gotz (1986) suggests a biphasic type of encapsulation exists with GRs reacting to the foreign surface and PLs reacting to the disintegrating GRs. This favours the concept that the stimulus is GR-derived and not from the foreign object.

1.3.2 Humoral immune responses

The haemolymph of insects contains both naturally occurring and inducible humoral defence factors: the nature and distribution of which has been extensively reviewed (Chadwick and Aston, 1978; Boman and Hultmark, 1987).

1.3.2.1 Humoral Encapsulation

This involves the deposition of a quickly hardening substance on the surface of foreign objects without the visible involvement of the cellular components of the host immune system. Such encapsulation has only been observed in larvae of insects with low blood cell counts such as Diptera and Hemiptera (Gotz and Vey, 1986). It serves to act as an efficient defence reaction against nematodes, fungi and bacteria. Of the fungi tested, *B. bassiana* has been shown to overcome humoral encapsulation (Gotz and Vey, 1974). Humoral encapsulation is the only defence reaction of *Chironomus* larvae against bacteria (Gotz *et al.*, 1987). Certain bacteria pathogenic to some insects (e.g. *S. marcescens, P. aeruginosa*) were found to be non-virulent in *Chironomus* larvae. This was due to the prevention of bacterial growth by humoral encapsulation.

Poinar and Leuteneggar (1971) described a biphasic formation of the melanized sheath around the nematode *Neoaplectana carpocapsae* in larvae of the mosquito, *Culex pipiens*. Within 25 min of invasion, a homogeneous deposit with low electron density was found surrounding the parasite. After 1 h electron dense granules appeared within this layer adjacent to the nematode surface. After 5-10 h, a definite capsule was observed, composed of an inner region with electron dense material, a non-melanized middle layer and an outer layer that contained cellular debris. Humoral encapsulation occurs in 2 steps; first the freshly attached capsule material is soft and colourless but then solidifies and changes to a brown/black colour. Capsule material of the first phase contains activated PO but during solidification the capsule gradually loses enzyme activity. Concomitantly, the electron density of the capsule material increases and tyrosine disappears from the haemolymph. Humoral encapsulation is controlled by various factors including ß-1,3-glucans, bivalent

ions (calcium) and a serine protease (Gotz and Vey, 1986). These findings are important as these factors also control activation of the pPO cascade.

1.3.2.2 Humoral factors

Many of the factors occurring in the haemolymph are often described separately from those arising following wounding or infection. However, such a division may be subject to an author's personal preference, e.g. agglutinins are naturally present in many insect species and can be absent or induced in others (Kubo *et al.*, 1984) or lysozyme, which also occurs naturally, can also have its titre increased after infection (Anderson and Cook, 1979). The humoral factors that have been implicated in defensive roles include lymphokine-like substances, components of the pPO cascade, agglutinins and antimicrobial proteins.

1.3.2.3 Lymphokine-like substances

A lymphokine is a substance that is "a non-antibody protein produced by activation of lymphocytes, which acts as an intercellular mediator of the immunological response" (Dumonde *et al.*, 1982). Invertebrates have not been shown to produce lymphokines. However, there are a number of factors which mediate immune processes and are referred to as 'lymphokine-like' (Ratcliffe *et al.*, 1985). Such factors include the 'injury factor' of wounded Lepidopteran pupae (Cherbas, 1973) or locusts (Gunnarsson, 1987), 'phagocytosis-stimulating factors' from waxmoths (Mohrig and Schittek, 1979) and cockroaches (Ratcliffe and Rowley, 1983), the 'plasmatocyte-depletion factor' of waxmoths (Chain and Anderson, 1983b) and 'encapsulation-promoting factors' of various arthropods (Ratner and Vinson, 1983).

The injury factor described by Cherbas (1973) was found in pupae of the saturniids *Samia cynthia, Antheraea pernyi* and *Hyalophora cecropia.* It was found to be released into plasma by damaged epidermal cells. Furthermore, it was also observed that wounding caused the PLs to become amoeboid and adhesive which assisted in wound healing. This factor was termed haemokinin and did not activate the pPO system. Bohn (1977) describes a haemocyte derived conditioning factor released upon wounding which influences the growth of the epidermal cells. The author suggests that this factor may be identical to the coagulogen released from the COs. Gunnarsson (1987) has shown that the haemocytes of infected locusts exhibited a decreased adhesiveness to glass with a concomitant increase in phagocytosis and nodule forming ability.

Phagocytosis-stimulating mediators have been reported to be present in *G. mellonella* (Mohrig and Schittek, 1979; Mohrig *et al.*, 1979) and in *P. americana* (Ratcliffe and Rowley, 1983). In the former, it was found that the normally non-phagocytosable cells of *B. thuringiensis subtoxicus* could be ingested by the haemocytes if the insects were simultaneously injected with latex beads. It was suggested that a phagocytosis-stimulating mediator was being produced by the latex bead injection because the active substance could be transferred to naive larvae in the plasma fraction of treated insects which activated the phagocytes to ingest the bacteria (Mohrig *et al.*, 1979) Ratcliffe and Rowley (1983) showed that preincubation of *P. americana* serum with a bacterial isolate, B1, resulted in enhanced phagocytosis rates in haemocyte monolayers. Gunnarsson (1987) observed that the number of phagocytically active cells increased after the injection of latex beads. This may also be brought about by a factor that stimulates phagocytosis

The 'plasmatocyte depletion factor' described by Chain and Anderson (1983b) was found in *G. mellonella.* They found that haemocytes released the factor after injection of *B. cereus* into *G. mellonella* larvae. The result of this

treatment was a rapid decline in the number of circulating plasmatocytes. Chain and Anderson (1983b) indicated that the plasmatocyte depletion factor was a humoral factor as it could be actively passed to non-immunized larvae of *G. mellonella* via the plasma. This factor was suggested to deplete the number of plasmatocytes in order for them to attach to the haemocoel lining and migrate chemotactically to the regions of the body that require nodule and capsule formation (Anderson and Chain, 1986). Geng and Dunn (1989) observed that depletion of PLs was dose-dependant when *M. sexta* were injected with Gram positive or Gram negative bacteria. However, they noted that wounding did not elicit this phenomenon.

'Encapsulation promoting factors' have been suggested by many authors and arise from the degranulation of granular cells at the foreign body/Haemocyte interface (Ratner and Vinson, 1983; Davies *et al.*,1988). Davies *et al.* (1988) have characterised an encapsulation promoting factor from *Heliothis virescens* and found that it was a trypsin sensitive peptide. They are thought to induce the attachment and flattening of plasmatocytes to the developing nodule or capsule. However, pPO activation has been implicated in this role and it has been suggested that the factors referred to by Ratner and Vinson (1983) are produced by activation of the pPO system (Soderhall and Smith, 1986a).

Haemocyte-derived proteins from *Ceratitis capitata* have been shown to be responsible for the recognition of *E. coli in vivo* (Marmaras and Charalambidis, 1992). One protein of 47 kDa can form adducts with tyrosine derivatives generated by tyrosinase activity and attach to the *E. coli* surface (Marmaras *et al.*, 1994). When the *E. coli*-protein was overlaid on haemocyte monolayers, the bacteria were entrapped by haemocytes.

1.3.2.4 The prophenoloxidase activating system.

Phenoloxidases have long been implicated in host defence mechanisms due to the appearance of melanin following host wounding or infection (Soderhall,1982; Ratcliffe *et al.,* 1985). Phenoloxidases have been found in the haemolymph in a large number of insect species (see Soderhall and Smith, 1986a). However, there is some disagreement as to the location of the enzyme within the haemolymph. Some authors favour its presence in the haemocytes (Schmit *et al.*, 1977; Leonard *et al.,* 1985a; Mills *et al.*, 1968; Preston and Taylor, 1970; Durrant *et al.*, 1993). On the other hand, PO has also been localised in the plasma or serum of other insects (Ashida *et al.,* 1982; Bidochka *et al.*, 1989; Saul and Sugumaran, 1987; Dunphy, 1991). In the haemolymph, POs are present as inactive precursors, prophenoloxidases (pPO), which can be activated by an enzyme from the cuticle (Ashida *et al.*, 1974) and possibly from the haemocytes (Leonard *et al.*, 1985b).The process of pPO activation to PO comprises of a cascade system (Soderhall and Smith, 1986b; fig.1) which has been compared to the alternative pathway of the complement system in vertebrates (Ashida *et al.*, 1983; Soderhall, 1982). The pPO activation system is complex and it is difficult to determine exclusively whether it is a cell- or plasma-associated system (Ratcliffe *et al.*, 1985). It would appear from the literature that the system comprises of both haemocyte- and plasma- derived factors.

It is because the release of the pPO system components is probably instantaneous that has led to the classification of the system as being a humoral component of the immune response (Ratcliffe and Rowley, 1987). This has raised some objections and, therefore, the classification of the agglutinins and antimicrobial proteins, which possibly rely on the haemocytes for release or synthesis must follow the same rule.

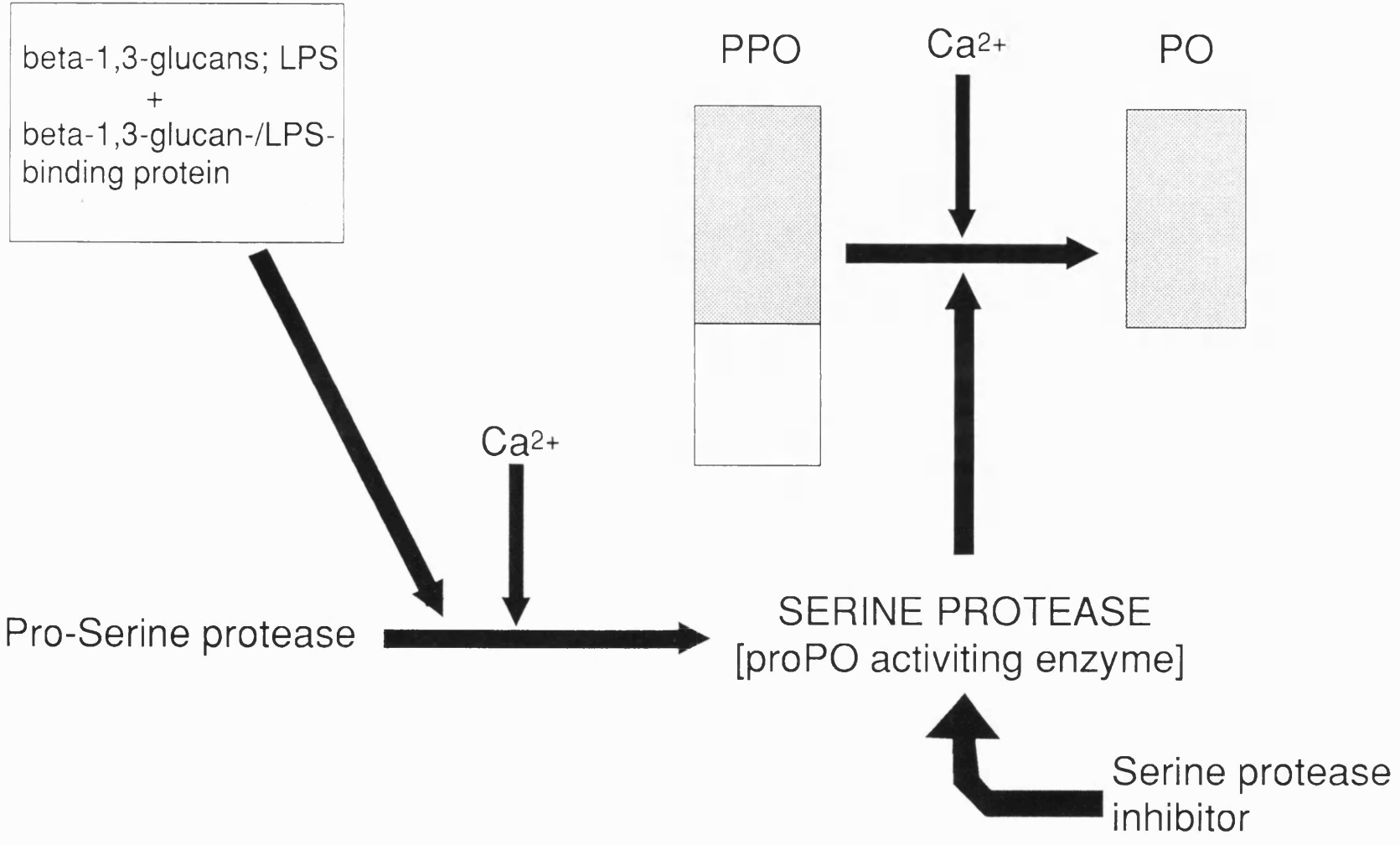


Figure 1. The prophenoloxidase cascade system in insects (after Soderhall and Smith, 1986b)

Much of the work performed on pPO and its activation mechanisms has used crustaceans as model systems (Johansson and Soderhall, 1989a). Crustaceans are marine arthropods and it has been speculated that the pPO activating system in crustaceans is not unlike that for insects and therefore, one can make direct comparisons between the two (Ashida and Soderhall, 1984; Ratcliffe and Rowley, 1987; Soderhall *et al.*, 1986) The pPO system is localised in the granular and semigranular cells of the crustacean haemocytes (Soderhall and Smith, 1983) and in the plasma of *B. mori* (Ashida *et al.*, 1982) and *H. cecropia* (Andersson *et al.*, 1989). Leonard *et al.* (1985a) report that the enzyme system is present in the haemocytes of the cockroach *B. craniifer.* The authors used an anticoagulant to reduce the possibility of rupture of the haemocytes and thus liberation of the pPO system. However, Ashida and colleagues used a cane sugar factor to 'stabilise' the pPO system. This factor decreased the total haemocyte count which would mean that any conclusions as to the whereabouts of the pPO system are invalid (Ratcliffe and Rowley, 1987).

Phenoloxidase (EC 1.10.3.1) catalyses the oxidation of tyrosine derivatives such as dihydroxyphenylalanine to quinones which spontaneously cross-react with tyrosyl residues on other proteins resulting in a hard insoluble substance called melanin (Andersen, 1985). The observation that melanization occurs after wounding or infection has been known for some time (Taylor, 1969; Nappi, 1973; Pye, 1974) leading to the inference that the pPO system is involved in immune function. Melanization can be inhibited by phenylthiourea (PTU) or reduced glutathione. Injection or feeding of these compounds to insects can prevent or reduce host encapsulation responses (Vey, 1979; Brewer and Vinson, 1971). However, Salt (1956) showed that whilst PTU prevented humoral encapsulation of *Nemeritis canescens* eggs, cellular encapsulation was unaffected. An explanation of these conflicting reports can be offered by observations from crustaceans (Soderhall and Smith, 1984). The authors showed that components of the pPO system, prior to melanization, were the

mediators of the immune response. Therefore, PTU may inhibit melanization but not activation of the capsular response. Soderhall and colleagues have provided evidence for the involvement of pPO in eliciting immune responses in crustacean haemolymph. Smith and Soderhall (1983) overlaid crustacean haemocyte monolayers with the bacterium *Moraxella* sp. suspended in a ß-1,3-glucan solution, glucose dextran, cellulose, chitin or saline. They found that phagocytosis of the bacteria was only significantly enhanced in the presence of the ß-1,3-glucan but not the other carbohydrates. Supplementary to this, Soderhall *et al.* (1984) coated fungal spores with the products of pPO activation and injected them into crayfish and allowed encapsulation to occur. Stronger encapsulation was observed with treated spores than with saline incubated controls. In insects, Ratcliffe's group (Ratcliffe *et al.*, 1984; Leonard *et al.*, 1985a) have provided evidence that the pPO system is involved in insect immune responses using *G. mellonella, B. craniifer* and *Leucophaea maderae* as models. The effects of ß-1,3-glucans, dextran, LPS and a serine protease inhibitor were tested on the phagocytosis of a *Bacillus cereus* and pPO activation. Both the ß-1,3-glucans and LPS enhanced phagocytosis (Ratcliffe *et al.*, 1984; Leonard *et al.*, 1985b) but the dextran- and protease inhibitor-treated insects did not. Furthermore, ß-1,3-glucans promoted the production of pPO. However, LPS did not stimulate pPO activation which indicates that some other factor(s) may be required. Brookman *et al.* (1989b) have shown that peptidoglycan and the capsule of some bacterial species can activate pPO in several insect species.

Our understanding of the biochemical events that occur during pPO activation in Arthropods comes from Ashida's group (Ashida *et al.*, 1982; 1983) and Soderhall and co-workers (Soderhall, 1992; Soderhall and Smith, 1986a,b; Johansson and Soderhall, 1989b) who worked on the silkmoth *B. mori* and the crayfish, *Astacus astacus* respectively. In both groups, pPO was found to be activated through a complex enzyme cascade which probably involves

several serine proteases. Phenoloxidase is a 'sticky' enzyme (Soderhall *et al.*, 1979) and its generation yields a number of additional sticky proteins (Ashida and Dohke, 1980; Soderhall *et al.,* 1979). The pPO cascade can be activated by a variety of microbial components such as ß-1,3-glucans (Unestam and Soderhall, 1977; Soderhall and Unestam, 1979) and peptidoglycan (Brookman *et al.*, 1989a) which are components of fungal and bacterial cell walls respectively. However, LPS has been found to stimulate the pPO cascade in crustaceans (Soderhall and Hall, 1984) but not in *B. mori* (Ashida *et al.*, 1983). Brookman *et al.* (1989a) have found that *E. coli* strains with differing LPS compositions influenced the strength of immune response in *L. migratoria* and *S. gregaria.* However, the authors were unable to draw any clear conclusions regarding LPS activation of the cascade. Another possibility is that arachidonic acid can activate the pPO cascade (Sugumaran and Kanost, 1993). Cellular damage could result in the release of phospholipase A_2 which, in turn, liberates lysolecithin and free fatty acids. The latter may be used for the synthesis of arachidonic acid and eicosanoids which would trigger further immune responses (Stanley-Samuelsson *et al.*, 1991).

Prophenoloxidase activation is the result of a cascade of reactions which amplify the original signal. Prophenoloxidase is activated by a pPO activating enzyme which is a serine protease and has been purified from the haemolymph of *P. leniusculus* (Aspan *et al.*, 1990). The pPO activating enzyme is, in turn, activated by an unknown mechanism involving ß-1,3-glucans and/or LPS (Soderhall and Smith, 1986b). Duvic and Soderhall (1990) have purified a ß-1,3-glucan-binding protein from the plasma of *P. leniusculus*. This protein was found to have remarkably similar physico-chemical properties to the binding proteins isolated from *B. craniifer* and *B. mori* (Soderhall *et al.*, 1988; Ochai and Ashida, 1988). Indeed, the amino acid compositions were similar indicating that the crustacean and insect proteins may be highly conserved. When incubated with laminarin, all three of these proteins were shown to

enhance PO activity. It has been suggested that, should the ß-1,3-glucan binding-protein be involved in the early stages of PO activation, then the components of the pPO activating system are present in the cells and not the plasma (Soderhall, 1992). This would seem important in regulating the activation of the enzyme which has been found to be present in the plasma of several arthropods. Furthermore, a LPS-binding protein has been identified in *P. americana* (Jomori *et al.*, 1990). In crustaceans, the pPO system can be released from semi-granular cells by microbial polysaccharides such as LPS and ß-1,3-glucan whilst release of the system from the granular cells can occur by either a 76 kDa factor or by the ß-1,3-glucan-binding protein if previously treated with a ß-1,3-glucan such as laminarin (Barracco *et al.*, 1991).

The protein with a molecular weight of 76 kDa has been associated with the pPO system and has been purified from the semigranular and granular cells of *P. leniusculus* (Johansson and Soderhall, 1988). It was found to stimulate encapsulation (Johansson and Soderhall, 1989a) and may be one of the 'encapsulation promoting factors' described earlier. The protein triggers degranulation of haemocyte monolayers (Johansson and Soderhall, 1989b). The protein's biological activities are generated outside the cells in the presence of ß-1,3-glucans and LPS. Thus, it is simultaneously activated with pPO (Johansson and Soderhall, 1989b) and may be activated by the same proteases or by-products of pPO activation. Low calcium ion concentrations also stimulate activation of the 76 kDa protein. The peptide sequence GRGDS mimics the effects of this protein. This is interesting because the sequence RGD is known to be responsible for the binding of cells to a number of vertebrate adhesion proteins (Johansson and Soderhall, 1989a; Ruoslahti and Piersbaucher, 1987)and mediates the encapsulation of foreign implants in the moth, *Pseudoplusia includens* (Pech and Strand, 1995). A protein in the blood of *B. craniifer,* antigenically related to the 76 kDa crustacean protein also induces degranulation of insect cells (see Johansson and Soderhall, 1989a).

Leonard *et al.* (1985a) and Soderhall (1981) have reported that the serine proteases responsible for the conversion of pPO to PO are present in the haemocytes. Soderhall and Hall (1984) have shown that there are two different serine protease-mediated pathways for activation of the pPO cascade depending upon whether ß-1,3-glucans or LPS is the activator. Leonard *et al.* (1985a) found that activation of pPO by ß-1,3-glucans required calcium ions. The pPO cascade can also be activated by low concentrations of calcium or pH changes (Soderhall, 1981). These observations are of importance as calcium ions initiate haemolymph coagulation. Of major interest is the fact that, in the presence of fatty acids, the entomopathogenic fungus *B. bassiana*, was found to leak calcium ions in vitro (see Gillespie, 1991). Therefore, in the absence of microbial activators, as in the case of wounding, low levels of calcium may be the only mechanism available for the host to initiate coagulation.

It is necessary to regulate the activation of the pPO activating system by regulating the proteinases present in the system. This will prevent uncontrolled melanisation which would otherwise be unnecessary or detrimental to the host. A number of different types of protease inhibitor have been identified in insects and crustaceans and will be discussed later (see chapter 4).

1.3.2.5 Agglutinins

Agglutinins, or lectins, are ubiquitous, non-immunoglobulin protein, or glycoprotein molecules found in many biological systems (Sharon and Lis, 1989) which bind to specific carbohydrate structures, and are frequently detected by their capacity to agglutinate cells (Vasta and Marchalonis, 1984). Haemagglutinins have been found in the haemolymph of numerous insects (Hapner, 1983; Yeaton, 1981; Richards *et al.*, 1989). These humoral substances are capable of agglutinating bacteria (Scott, 1971), protozoa (Jurenka *et al.*, 1982; Ingram *et al.*, 1984) and cestodes (Lackie, 1981). Lectins have also been

found on the surfaces of the cestode *Hymerolyptis diminuta* (Lackie, 1981) as well as several entomopathogenic fungi (Pendland and Boucias, 1986). These observations have led to the assumption that invertebrate lectins serve to opsonise and/or clump invading parasites, facilitating their encapsulation and phagocytosis (Rowley *et al.*, 1986). In the mollusc, *Mytilus edulis*, Renwrantz and Stahmer (1983) have shown an opsonic role for lectins. The site of synthesis of lectins has been suggested to be the fat body (Komano *et al.*, 1983) and the granular and/or spherule cells (Amirante, 1976).

There is some evidence for a role for lectins in the immune response. Komano *et al.* (1980; 1981) and Kubo *et al.* (1984) have found that, 48 h after injury of the body wall of the flesh fly, *Sarcophaga peregrina*, haemagglutinating activity in the haemolymph increased. Komano *et al.* (1981) reported that the lectin from injured larvae consisted of α and ß subunits whilst that from uninjured larvae contained only the a subunit. The authors postulated that, following injury, some of the α subunits were converted to ß subunits by limited proteolysis to yield the active lectin. This finding is extremely important when we consider lectin involvement in the immune response. The naive pupal lectin, in its a subunit form cannot act on self components. Ratcliffe and Rowley (1987) indicate that there may be a possible interactive relationship between the pPO cascade and the agglutinins because, following activation of the pPO system, serine proteases are released from the haemocytes which may cause partial proteolysis of the α subunit of the lectin. Furthermore, Komano *et al.*, (1980) believe that the *S. peregrina* lectin enhances phagocytic activity of the PLs to clear debris tissue following pupation or invading foreign substances.

1.3.2.6 Antimicrobial factors

These factors consist of naturally occurring and inducible factors present in the haemolymph of insects. They include lysozyme (Powning and Davidson, 1973; Zachary and Hoffmann, 1984), non-lysozymal bacteriocidins (Kinoshita and Inoue, 1977), lysosomal enzymes (Ratcliffe and Rowley, 1979), lysins (Gingrich, 1964; Dunphy and Nolan, 1980) and antimicrobial proteins (Boman and Hultmark, 1987; Hultmark, 1993).

Lysozyme (N-acetylmuramide glucanohydrolase) occurs naturally in insects but can also be induced by prior immunisation (Chadwick, 1970; Powning and Davidson, 1973; Anderson and Cook, 1979). The enzyme is a hydrolase and cleaves the 1,4 linkages between N-acetylglucosamine (GlcNAc) and N-acetylmuraminic acid which are present in the cell walls of Gram positive bacteria. Chadwick (1970) has shown that there is a parallel development of lysozyme levels and protective immunity in *G. mellonella* after immunisation with *P. aeruginosa.* Anderson and Cook (1979) have demonstrated the existence of lysozyme-like activity in the blood cells of *Spodoptera eridania.* Furthermore, they showed that injection of *E. coli* LPS stimulated an increase in levels of lysozyme within the haemocytes. Dunn *et al.* (1985) have shown that lysozyme increased following the injection of bacterial peptidoglycan. This increase was also shown to be dependant upon synthesis of RNA and to involve the synthesis of new protein. Zachary and Hoffmann (1984) have isolated lysozyme from the haemocytes of *L. migratoria* and shown that it is in the granules of the GRs and COs. They inferred that lysozyme is released as a result of coagulation. Therefore, lysozyme would be released at the sites of wounds to prevent bacterial invasion. It would also be released at foreign object/haemocyte interfaces which may aid in microbicidal activity at the centre of nodules and capsules. Hultmark *et al.* (1980) have sequenced the lysozyme from *Hyalophora cecropia* and found that it has great

similarity with vertebrate lysozymes of the chicken type. The fact that LPS can stimulate lysozyme synthesis and not activate the pPO system in insects suggests that there may be a selective activation of components of the immune system. Bacteria can stimulate antibacterial factors whilst larger organisms activate pPO allowing encapsulation and melanization to occur.

Lysozyme is only effective against Gram positive bacteria but Kinoshita and Inoue (1977) have indicated that *B. mori* haemolymph contains a cofactor which allows lysozyme to work against Gram negative bacteria. However, no work has been performed on its mode of action. Russell and Dunn (1990) have indicated that lysozyme levels increased moderately in homogenates of pericardial cells after injection with bacteria. This would imply that the pericardial cells are also involved in the immune responses. Schneider (1985) has purified 3 lysozymes from the cricket, *Gryllus bimaculatus* which could be induced with a sub-lethal dose of *B. thuringiensis*. He could find no other antibacterial proteins present in the haemolymph and suggested that lysozyme is the only bacteriolytic component of the cricket and other hemimetabolic insects. Only holometabolic insects have been shown to contain antibacterial proteins that have a broader spectrum of antibacterial activity than lysozyme. The author indicates that hemimetabolic insects differ in their development and different defence mechanisms against bacterial infections may be expected. Lysozyme has also been shown to be induced in response to injection with bacteria (Azambuja and Garcia, 1987).

Enzymes such as acid phosphatase and ß-N-acetylglucamidase which hydrolyse phosphate esters and peptidoglycan in bacterial membranes and walls respectively, have been found to occur naturally in the Haemocytes of *G. mellonella* and *M. sanguinipes* (Ratcliffe and Rowley, 1979; Miranpuri *et al*, 1991). Furthermore, the activities in the former were observed to become greatly enhanced after the injection of bacteria (Ratcliffe and Rowley, 1987).

The ramifications of these findings are that these enzymes would also contribute to the breakdown of the microbial cells at the site of wounding.

Other antimicrobial factors found in insects include a lysin from *Oncopeltus fasciatus* which exhibits inhibitory activity against *P. aeruginosa* (Gingrich, 1964). There is also evidence for a protozoan lysin in the plasma of the spruce budworm *Choristoneura fumiferana* (Dunphy and Nolan, 1980).

1.3.2.7 Immune proteins

Aside from the naturally occurring enzymes such as lysozyme and the lysosomal enzymes, various inducible antimicrobial proteins exist in insect haemolymph (Boman and Hultmark,1987). The pioneering work on inducible proteins was carried out by Boman and co-workers using diapausing pupae of the giant silkmoths, *Hyalophora cecropia, Samia cynthia* and *Antheraea pernyi* as their models. The advantage of using these insects lies in the fact that they are generous in haemolymph volume and diapause results in low metabolic activity so that, following injection, only the genes involved in immune protein production are turned on. Thus it was easy to selectively label the RNA and proteins formed by these genes.

Immune proteins first made an appearance in the haemolymph after a lag phase of 8-10 hours and peaked at 7-8 days after infection (Gotz and Boman, 1985). Actinomycin D and cycloheximide inhibited the production of these immune proteins indicating *de novo* synthesis of RNA and proteins are required. After separating radioactively labelled immune haemolymph on polyacrylamide gels, 9 protein bands (designated P1-P9) were found. The major immune proteins formed were P4 (hemolin) and P5 proteins. Immune protein P7 was found to be lysozyme (Hultmark *et al.*, 1980) based on its similarities in amino acid composition and bacteriolytic properties. Hemolin was the largest protein (48 kDa) synthesised but was notfound to perform any function (Gotz

and Boman, 1985). Kanost and Ladendorff (1990) isolated a protein from *M. sexta* that is homologous to *H. cecropia* P4 protein. The concentration of this protein was found to increase after the injection of Gram positive or Gram negative bacteria but not of saline or protein alone. This protein was sequenced and an open reading frame generated. This revealed that the protein is homologous with members of the immunoglobulin superfamily and was similar to the C2-type immunoglobulin domain (Sun *et al.*, 1991a). The hemolin protein has subsequently been found to have an opsonic role in the immune function of *M. sexta* as it binds to bacterial surface molecules and forms a stable complex with other haemolymph proteins (Sun *et al.*, 1990). The specificity of the binding to bacteria is not clear, but when using *E. coli* mutants that lack sugars in the LPS core, the complex formation between hemolin and haemolymph proteins was abolished (Schmidt *et al.*, 1993).

P5 protein was found to be composed of at least 6 distinct proteins (A-F) each having a molecular weight of 21-23 kDa (Hultmark *et al.*, 1983). These proteins have been named attacins and are divided into 2 groups according to their amino acid composition and amino-terminal residues; the basic (attacins A-D) and the acidic (attacins E-F). The proteins are synthesised as inactive precursors, preproattacins which have N-terminal extensions (Lee *et al.*, 1983). The antibacterial activity of the attacins has been demonstrated by overlaying a polyacrylamide gel with bacteria. They were found to be powerful antibiotics at physiological concentrations (Engstrom *et al.*, 1984). By using different bacterial isolates, it was shown that the attacins were narrow spectrum antibacterial proteins that killed the Gram negative bacteria *E. coli, Pseudomonas maltophila* and *Acinetobacter calcoaceticus*. Furthermore, an *E. coli* mutant D11, which was division defective was found to be considerably less sensitive than the normal parental strain indicating that the attacins act on growing cells (Engstrom *et al.*, 1984). Furthermore, the penetration of the outer membrane of *E. coli* by ß-lactam antibiotics, Triton X-100 and chicken

egg white lysozyme-compounds that are normally inactive against *E. coli* alone due to their inability to penetrate the outer membrane, was facilitated by the addition of attacins.

The final and most active group of antimicrobial immune proteins was the P9 group which were called cecropins. The cecropins constitute a family of proteins with molecular weights of approximately 4 kDa and possess a broad spectrum of activity against Gram negative and Gram positive bacteria. Six distinct cecropins (A-F) have now been identified as well as a factor G which may also be a cecropin (Steiner *et al.,* 1981; Hultmark *et al.*, 1982). The antibacterial activity of the cecropins has been assessed (Hultmark *et al.*, 1980; Steiner *et al*, 1981). Cecropins A and B have a broad range of activity against both Gram positive and Gram negative bacteria (Hultmark *et al.*, 1982). Engstrom *et al.* (1984) found that the sensitivity of *E. coli* to cecropin B was potentiated with the addition of attacins.

In addition to the cecropins, antibacterial proteins have now been identified in *G. mellonella* (Hoffmann *et al.*, 1981), *M. sexta* (Dickinson *et al.*, 1988; Hughes *et al.*, 1983), *Apis mellifera* (Casteels *et al.*, 1989), *Drosophila melanogaster* (Flyg *et al.*, 1987; Hoffmann *et al.*, 1993), *S. peregrina* (Okada and Natori, 1983) and several other orders (Gotz and Trenczek, 1991. These proteins are referred to as 'cecropin-like' (Flyg *et al.*, 1987; Kaaya *et al.*, 1987) or given new names such as sarcotoxin (Okada and Natori, 1985), gallysin (Phipps *et al.,* 1994), drosocin (Hoffmann and Hetru, 1992) or diptericin (Keppi *et al.*, 1986).

The major cecropins are A, B and D and have been purified and sequenced (Steiner *et al.,* 1981; Hultmark *et al.*, 1980). Cecropins B and D from *A. pernyi* have been also been sequenced (Qu *et al.*, 1982) and show such a homology with those from *H. cecropia* that it has been suggested that the proteins have arisen due to gene duplication from a common ancestral gene (Boman and Hultmark, 1987). Model building from amino acid sequences has

indicated that the cecropins can form near perfect amphipathic α-helices (Andreu and Merrifield, 1985; Boman *et al.*, 1991). Proteins containing these helices are often associated with membranes and the predicted secondary structure may be of importance for the membrane disrupting activities of the cecropins. Faye *et al.* (1975) have suggested that this system is similar to complement in that both membrane attacking complexes involve the interaction of 7-8 polypeptide chains, lipid A inhibits the activity of both systems and that osmotic pressure within the bacteria contributes to the killing by the two defence mechanisms.

Christensen *et al.* (1988) have shown that the cecropins A, B and D as well as synthetic analogues form large time-variant and voltage dependent ion channels in planar lipid membranes. The authors propose a model for cecropin action: (a) the cecropin oligomers interact with the membrane bilayer via electrostatic adsorption; (b) the hydrophobic C-terminal region is inserted into the hydrophobic membrane core; and (c) upon application of a voltage, a major conformational rearrangement takes place which results in channel formation. This results in the insertion of the positively charged amphipathic α-helix in a membrane-spanning configuration with the charged residues forming the inner water-filled pore. The net result is cell lysis and death. The authors believe that this mode of action also applies to the sarcotoxins and magainins. Cecropins have been identified in the small intestine of pigs (Lee *et al.*, 1991) indicating a role for the antibacterial proteins in protection in higher mammals.

However, Hoffmann and colleagues have suggested a different mode of action for a defensin, diptericin A, isolated from *Phormia terranovae* (Keppi *et al.*, 1989). Diptericin A differs from cecropins and attacins in that it is a basic protein with a molecular weight of 8.6 kDa (Dimarq *et al.*, 1988). Significantly, there is no evidence that the diptericin A molecule is capable of forming an α-helix. Furthermore, it is also only effective against a limited array of Gram-negative bacteria. Keppi *et al.* (1989) suggested that diptericin A acts at the

level of the cytoplasmic membrane leading to lysis. Their action results in amino acid transport inhibition after 2 minutes and decreased bacterial cell viability after 30 minutes. After 1 hour cytoplasmic ß-galactosidase is released and finally the cell wall disintegrates as indicated by the release of diaminopimelic acid.

Another distinct group of antibacterial proteins, the defensins (Lambert *et al.*, 1989) affect Gram positive bacteria and appear to be present in Diptera, Coleoptera, Hemiptera and Odonata (Hoffmann and Hetru, 1992). These proteins are also ubiquitous in mammalian systems (see Lehrer *et al.*, 1993) especially in phagocytes and neutrophils. In mammals, they participate in non-oxidative microbicidal mechanisms via membrane permeabilisation of ingested microrganisms. However, unlike insect defensins, they are not secreted into the cell-free blood, probably because they lack sequence homology. To date, 11 defensins have been characterised from various insect sources (see Cociancich *et al.*, 1994) but their mode of action remains to be elucidated.

Natori's group have found that larvae of the flesh fly, *S. peregrina* produced three bands of antibacterial proteins termed sarcotoxins, after injury (Natori, 1977). They also found that two of these bands, when purified, each contained three proteins (Okada and Natori, 1983; Ando *et al.*, 1987). The first group to be purified were that of the sarcotoxin I family. It was found that each protein was 39 amino acids long and each differed by two or three amino acid residues (Okada and Natori, 1984). The action of sarcotoxin I was shown to be directed toward the bacterial membrane (Okada and Natori, 1984). Ando *et al.* (1987) purified the sarcotoxin II group and found three proteins (sarcotoxins IIA, IIB and IIC) with almost identical primary structures. Antibodies raised to sarcotoxin IIA cross-reacted with the other two members of the group indicating a high degree of structural relatedness. Furthermore, Sarcotoxins I and II are completely different proteins with different molecular masses. However, the biological significance of the isomeric forms of sarcotoxins I and

II remains unclear but it was speculated, in light of the observations that cecropins and attacins are present as isoforms, each isoform may have a different antibacterial spectrum (Ando *et al.*, 1987). In addition, these proteins may interact to obtain the desired result of cell death.

Thus, the net result of either cecropin, sarcotoxin or diptericin A activity is cell lysis It would appear that there are a variety of antibacterial proteins present in different species of insect and that they possess different modes of action yet achieve the same result-bacterial death. Brey *et al.* (1993) have shown that the epithelial cells underlying the cuticle synthesised antibacterial proteins after the epicuticle had been lightly abraded in the presence of bacteria or bacterial cell wall components. The production of antibacterial proteins has been well documented but in response to the injection of bacteria. In nature, insects are far more likely to receive a topical scratch than a puncture wound. Interestingly, the antibacterial protein found (cecropin) was not found in the underlying haemolymph or the unabraded portion of the cuticle. It is possible that this localised response is one analogous to the inflammatory response observed in mammals

All of the above findings have been made using bacteria as the microbe to challenge the immune system. Iijima *et al* (1993) have subsequently purified a protein from the blood of 3rd instar *S. peregrina* that can kill the yeast, *Candida albicans* but not repress the growth of bacteria. Furthermore, there was a significant synergism between the antifungal protein and Sarcotoxin 1A. Of even more interest, the antifungal protein was shown to bind to *C. albicans* and cause leakage of an, as yet, unidentified substance. This novel protein present in high quantities (250 μg/ml) and speculation exists as to whether it has other physiological functions.

The induction of the immune proteins in insects presents an interesting case of gene regulation. By comparing the upstream regions of the *H. cecropia* immune genes, it appeared that the genes for basic and acidic attacins had in

their promoter regions a sequence similar to the mammalian transcription factor NF-κB (Sun *et al.*, 1991a). The importance of the κB site is that it is the enhancer of the immunoglobulin κ chain gene (Sen and Baltimore, 1986). mammalian genes that contain these regions are involved in the immune inflammatory or acute phase response (see Faye and Hultmark, 1993). Subsequently, similar sequences have been found in the lysozyme and cecropin A and B genes from *H. cecropia* and the *Drosophila* cecropin and diptericin loci (see Faye and Hultmark, 1993).

To test the functional significance of the κB motif, DNA-protein binding assays were performed using a fragment from the upstream region of the lysozyme gene which included the κB-like site. This fragment bound a nuclear factor from an extract of bacteria-stimulated pupae. Furthermore, upon removal of the κB-like site, binding was abolished (Sun *et al.*, 1991b). Because this factor was absent in naive pupae, it became named the Cecropia Immunoresponsive Factor (CIF; Sun and Faye, 1992a). The binding of purified CIF to the attacin κB motif could be abolished using antibodies raised against the lysozyme NF-κB sequence (Sun and Faye, 1992b). This shows the strong degree of homology between the two factors. Further understanding of the regulation of these genes is required in order to manipulate the properties of their protein products.

The importance of antibacterial peptides in human infectious disease is immense. These proteins have been shown to be active against the parasites that cause malaria (Boman *et al.*, 1989), Chagas' disease and leishmaniasis (see Kimbrell, 1991). Furthermore, antibacterial peptides may eventually be used as viable antibiotics in human healthcare (Kimbrell, 1991).

1.4. Pathogenesis of Entomopathogenic fungi.

There are over 700 species of fungi that are entomopathogenic. With regards to their pathogenesis, most information is known about the disease

caused by *Metarhizium* spp. and *Beauveria* spp. The following is a review of the process of pathogenesis primarily by these two species.

Mycoses is established according to a series of events which are outlined below. Attachment of a fungal spore to the host cuticular surface represents the initial event in the onset of mycosis (Charnley, 1984). In order to initiate infection, conidia must come into contact with the host cuticle for a period long enough for germination and subsequent hyphal invasion to occur. The mechanisms by which conidiospores attach to the cuticle include random association, hydrophobic association, agglutination and electrostatic interaction (Fargues, 1984; Boucias *et al.*, 1988). For most Deuteromycetes, attachment is passive and non-specific (Boucias *et al.*, 1988). In the case of *B. bassiana* vs. *Anticarsia gemmatalis,* hydrophobicity of the conidial wall and the insect epicuticle mediates the adhesion process.

B. bassiana and *M. anisopliae* both require exogenous carbon and nitrogen sources such as carbohydrate, amino acids and protein for germination (Smith and Grula, 1981, Dillon and Charnley, 1985; 1991). However, despite these non-fastidious requirements, nutrient availability on the surfaces of an insect's cuticle may prove limiting (Grula *et al.*, 1978). Woods and Grula (1984) found that water soluble nutrients such as amino acids on the surface of the corn earworm, *Heliothis zea*, were sufficient to induce *B. bassiana* germination. However, the same fungus will not germinate on sclerotised cuticle of the bark beetle, *Dendroctonus ponderosae* without a nutrient supplement (Hunt *et al.*, 1984). On the other hand, entomopathogens that have a restricted host range, may have more specific requirements for germination. For example, *N. rileyi*, primarily a Lepidopteran pathogen, responds to diacylglycerols and polar lipids (Boucias and Pendland, 1984). This is important as the cuticular surface of hosts is essentially made up of lipids. St. Leger *et al.* (1988a) have shown that *M. anisopliae* can grow *in vitro* on a variety of hydrocarbons including multi-branched alkanes which are refractory

to most fungi. Extracts from insect cuticle, which include short chain fatty acids (Koidsumi, 1957), have proved to inhibit the germination of *B. bassiana*.. However, Hunt (1986) found that none of the cuticular extracts from *D. ponderosae* inhibited germination. Smith and Grula (1982) found that conidial germination could be inhibited by the addition of C4:0 to C9:0 fatty acids to a 1% N-acetylglucosamine liquid media which otherwise induced excellent germination and growth. Bidochka (1989) showed that the short chain fatty acids are mycostatic and not mycocidal. Furthermore, the author found that the cuticle of *M. sanguinipes* contains C14:0 to C20:0 fatty acids which were not fungicidal to *B. bassiana* .

Humidity and temperature are other factors that can effect germination and growth of *B. bassiana*. Walstad *et al.* (1970) found that relative humidities greater than 92.5% were a requirement for conidial germination in agar media and humidity has often been cited as a limiting factor in mycosis (see St. Leger, 1991; St. Leger, 1993). However, this is not always the case (Ferron, 1977; Ramoska, 1984). In particular, the relative humidity was not a contributory factor to the pathogenesis of *M. sanguinipes* by *B. bassiana* (Marcandier and Khachatourians, 1987). Measurements of microclimate on cuticle have not been performed but the differences in microenvironment over the surface of insect hosts may explain the apparent conflicting reports of relative humidity in disease initiation.

The influence of cuticular components on the behaviour of entomopathogenic fungi has always been tested with extracts from non-infected intact insects. However, components secreted by the insect through the pore canals in response to the recognition of the presence of the fungal pathogen could also influence the germination of spores (Boucias and Latge, 1988). After germination, some entomopathogenic fungi will produce germ-tubes and appressoria (Charnley, 1990; Bidochka and Khachatourians, 1992). Germ tubes adhere to the epicuticle by an amorphous mucus which is secreted by the

hyphal tip (St. Leger *et al.*, 1988b). For many pathogens, it is not known what causes the cessation of germ tube elongation and the differentiation of penetrant structures which may or may not include appressoria. Appressoria are morphologically distinct swellings at hyphal apices formed on the cessation of germ tube elongation (Charnley, 1984). These structures assist in anchoring the fungus to the substrate but, also aid penetration by liberating enzymes (Zacharuk, 1970; St. Leger *et al.*, 1987a). Not all isolates of *B. bassiana* produce appressoria (Vey and Fargues, 1977; Pekrul and Grula, 1979; Bidochka and Khachatourians, 1992. However, a hold-fast structure is a consistent feature of *M. anisopliae.* Appressorial formation by *M. anisopliae* is induced preferentially over hair sockets of early instar larvae in *M. sexta* (St. Leger *et al.,* 1988b). In contrast, appressorial formation by the same isolate occurs much closer to the conidium on the cuticles of *Calliphora vomitoria, S. gregaria* and late instar *M. sexta* which lack the extensive microfolding of the epicuticle observed on early instar larvae. These observations could be replicated on polystyrene mimics of early and late instar *M. sexta* cuticle indicating that the inhibitory effects of microfolds are due to the surface topography rather than chemical differences (Charnley, 1989).

With some isolates of *M. anisopliae* on some cuticle types, surface growth appears extensively and may aid in the location of thinner or softer cuticle or enhance invasiveness via synergism with other hyphae (Charnley, 1984).

The next stage in infection is penetration. The main route of penetration through the cuticle is through the arthrodial membranes at joints and between segments, segmental cuticle and mouth parts. Using scanning electron microscopy, Pekrul and Grula (1979) revealed that the conidia of *B. bassiana* germinated and the resultant germ tubes penetrated the cuticle of *H. zea* within 18 h of exposure. A clean circular or oval hole was observed around the germ

tube at the penetration point of the cuticle. They also observed penetration of tracheal walls.

Indentation of host cuticle is indicative of mechanical penetration and holes around penetrant pegs suggest enzymatic degradation (Pekrul and Grula, 1979). *B. bassiana* and *M. anisopliae* synthesise extracellular enzymes such as proteases, chitinases, lipases and esterases when grown *in vitro* on ground insect cuticle (see Charnley and St. Leger, 1991; Khachatourians, 1992). Histochemical and ultrastructural studies, as well as enzyme extractions, have established that these enzymes are produced *in vivo* during host penetration (Zacharuk, 1981; Hassan and Charnley, 1989; Goettel *et al.*, 1989). Potential co-operation between enzyme types in the invasion of cuticle has been shown in model studies (St Leger *et al.,* 1987a). Samsinakova *et al.* (1971) observed complete disintegration of *G. mellonella* cuticle by commercial preparations of lipase, protease and chitinase in that order. Similar results were obtained by Smith *et al.* (1981) with *H. zea* cuticle. The importance of the prior action of proteases for efficient cuticle hydrolysis is reflected in the sequence of enzyme production by fungus *in vitro* and *in vivo.* Esterase and proteolytic enzymes (endoprotease, aminopeptidase and carboxypeptidase) are produced first followed by N-acetylglucosaminidase. Subsequent to this, chitinase and lipase are produced (see Charnley and St. Leger, 1991). The regulation of production of these enzymes is discussed in section 4.1.

Once a penetrant hypha enters the haemocoel, a number of options are available. Some entomopathogenic fungi continue filamentous growth (Yendol and Paschke, 1965). However, most entomopathogenic fungi convert to a yeast-like phase. The causes of death from mycoses are likely to vary with the behaviour of the pathogen. There is circumstantial evidence from Deuteromycete pathogens for the involvement of fungal toxins in host death (Roberts, 1980). Behavioural symptoms such as partial or general paralysis and decreased irritability in mycosed insects are

consistent with the action of neuromuscular toxins (Samuels *et al.*, 1988). Toxins have been shown to reduce encapsulation responses *in vivo* (Vey *et al.*, 1985) as well as nodule formation (Huxham *et al.*, 1989). Most work has been performed using destruxins from *M. anisopliae* but there have been suggestions for the involvement of *B. bassiana* metabolites such as the cyclodepsipeptides, bassianin, beauvericin, beauverilides and bassianolide in pathogenesis (see Roberts, 1980). Extensive growth in the haemolymph and penetration of host tissues will disrupt host physiology and cause stress reactions possibly including autointoxication (Charnley, 1984). Within a period of several days to several weeks, under conditions of high relative humidity, the fungus grows back out of the body wall of the cadaver, particularly at intersegmental membranes, and sporulates on the surface.

1.5. Aims of the Project.

The failure of modern synthetic chemicals to provide economic, environmentally friendly, efficient control of grasshoppers and locusts has led to a worldwide interest in the development of alternative biologically based forms of control. Foremost of these initiatives is the collaboration between the International Institute of Biological Control (IIBC), Ascot, the International Institute of Tropical Agriculture, Cotonou, Benin and the Department de Formation en Protection de Vegetaux (DFPV), Niamey, Niger in the development of fungal pathogens (principally *Metarhizium* spp.) for the control of acridid pests The desert locust, *Schistocerca gregaria*, is the main target for this work, though a number of West African grasshoppers are under investigation. An understanding of the mechanisms of fungal pathogenesis and the host immune response will help the development of more efficient locust-killing mycoinsecticides. In

the first instance, such work may provide a basis for rational strain selection and, in the longer term, identify targets for optimisation in a strain improvement programme.

This study is one of a number of projects at Bath aimed at an understanding of the relationship between *Metarhizium* spp. and the desert locust, *Schistocerca gregaria*. The focus of the present work is two-fold. Firstly, to determine the effects of mycosis on the blood-borne host defences of the locust (chapter 2) and secondly, to investigate the role of the cuticle-degrading chymoelastase, PR1, in isolate virulence (chapter 4). These two main aspects of the work come together in a study of inhibitors of PR1 in the blood of the desert locust. Problems encountered with this aspect of the work led to a subsidiary study of PR1 inhibition in the blood of the tobacco hornworm, *Manduca sexta* (chapter 3).

Chapter 2

2.0. The immune reactions of *S. gregaria* to the topical application of conidia of the entomopathogenic fungus, *Metarhizium* spp.

2.1. The immune reactions of locusts and grasshoppers.

Insect immune responses to microbial infection are the subject of intensive research. However, to date, the blood borne defences of Acridids, including *S. gregaria*, have been poorly characterised. Dularay and Lackie (1985) investigated the cellular encapsulation process in *S. gregaria* and its possible involvement in the prophenoloxidase (pPO) activation pathway. They observed that sepharose beads were not encapsulated *in vivo* by haemocytes after preactivation of the pPO pathway with calcium ions or zymosan (a β-1,3-glucan). From this, they suggested that there was no opsonic involvement of pPO in locust immune reactions. Huxham *et al.* (1989) showed that destruxins (cyclic depsipeptide toxins produced by *Metarhizium* spp.; Samuels *et al.*, 1988) decreased locomotion of locust haemocytes in monolayers, suppressed the laminarin- or zymosan-induced activation of haemocytes and inhibited the activation of pPO.

Lackie *et al.* (1985) have analysed the different cell types of *S. gregaria* haemolymph using Percoll density gradient centrifugation and observed 4 main types of haemocyte: plasmatocytes (PLs) which "spread extravagantly on contact with protein coated glass and contain large numbers of granules and vacuoles."; coagulocytes (COs) which "lyse or degranulate *in vitro* and may appear as either as a nucleus and remnants of surrounding cytoplasm attached to the slide by thin streamers of diffuse coagulum."; granular cells (GRs) which were "phase bright, granule

containing round cells anchored by thin filopodia." In addition, there was a small proportion of prohaemocytes (PROs) These cells were round, small and phase bright.

Gunnarsson and Lackie (1985) injected conidiospores of *M. anisopliae* or microbial cell wall factors (β-1,3-glucans) into the haemocoel of *S. gregaria* and observed that, after injection of whole spores, β-1,3-glucans (constituents of fungal cell walls) or lipopolysaccharide (LPS), there was a significant rise in the number of nodules. Furthermore, dextran, an α-1-6 glucan did not induce nodule formation. Upon injection of spores, large compact nodules were produced. However, after the injection of β-1,3-glucans or LPS, small and medium sized nodules were formed. Of interest was the observation that a 24 hour-old spore suspension induced significantly more, but smaller, nodules than a freshly harvested suspension. The reason why particles induced large nodules whereas soluble molecules induced small and medium sized nodules remains unexplained.

Gunnarsson (1988a) found an immediate decline in the total haemocyte count (THC) of the desert locust 10 minutes after injection of β -1,3-glucans (zymosan, laminarin). This decline was concomittant with the formation of nodules and recovered to half the original value after 10 hours and remained there. The remaining haemocytes were sensitized toward further challenges by injection. Twelve hours after an initial challenge with zymosan, the number of nodules observed was close to that for control insects, however, when challenged further with zymosan or saline, there was an increase in the number of nodules formed over and above the control. In addition, phagocytosis of sepharose beads was enhanced by prior injection of β-1,3-glucans due to an increase in the number of phagocytically competent haemocytes.

Gunnarsson (1988b) studied the cytology and the extent of the response of *S. gregaria* to physical wounding and penetration by *M. anisopliae*. The author found that, when the fungus was penetrating the cuticle, haemocytes were recruited to and attached to the basement membrane. Those haemocytes reacting at the wound site were different to those found underneath untreated cuticle. Furthermore, a structure similar to a capsule was formed around the site within 6 hours of infection. Of particular interest was the observation that the capsule was formed when the fungus had only penetrated as far as the epi- and exo-cuticle. It was not until the capsule had become melanized that hyphae grew through the remaining cuticle and melanized mass into the haemocoel. If the cuticle was scarified (not penetrated but scratched), the reaction observed with fungal penetration could be mimicked. However, if the cuticle was pierced with a needle, then a plug without the same conformity as the capsule was formed which was more localised. Gunnarsson (1988b) hypothesised that a wound factor was released during infection that recruited haemocytes to the site of infection. This inflammatory response probably initiated the decline in total haemocyte counts (THC) observed in insects topically infected with *M. anisopliae.* A reduced THC was first established, however, at the time when fungal hyphae were first found in the haemocoel (Gunnarsson 1987). Haemocytes that remained in circulation also had altered properties, including an increase in phagocytic potential. These results clearly show that the haemocytes are an integral component of the immune response of the desert locust to fungal infection. However, the cell types involved in these reactions were not identified by Gunnarsson.

Some work has also been performed on the blood-borne immune system of the migratory locust *Locusta migratoria*, by Ratcliffe and colleagues. They found that peptidoglycan, but not teichoic acid, elicited

nodule formation and activated pPO (Brookman *et al.* 1989a; b). Capsulated forms of *Klebsiella pneumoniae* had increased ability to induce nodule formation and activate pPO when compared to a strain that was acapsular. Various strains of *Escherichia coli* containing different amounts of LPS were tested and strains with the most LPS elicited the most nodules. As the number of oligosaccharides making up the LPS decreased, so did the bacteria's induction ability. However, when the same set of experiments were performed using *S. gregaria* as the source of haemolymph, the reverse situation was observed, i.e. the shorter the LPS became, the more nodules were formed. The same trends were observed in the two species of locust with respect to pPO activation. The net result, however, was conclusive proof for some sort of co-operation between pPO activation and nodule formation.

Some work has also been performed on the immunology of grasshoppers, particularly the migratory grasshopper, *Melanoplus sanguinipes*. Bidochka and Khachatourians (1987a) observed that *M. sanguinipes* responded to the injection of conidiospores of *B. bassiana* by decreasing the number of circulating haemocytes and increasing the number of melanotic nodules. Gillespie and Khachatourians (1992) observed that there were higher levels of PO in infected and wounded insects than in controls 10 minutes after injection. The difference between wounding and the injection of conidiospores was that in the latter case, a secondary response was observed. This phenomenon was not observed in insects that had been wounded, injected with protoplasts or inactivated spores. This suggests that the surface wall components of the fungal hyphal bodies (produced by germinating spores) elicited a response in the grasshopper.

Previous work on the immune response of locusts and grasshoppers to fungal infection is fragmentary and has often involved injection of

spores. This method of inoculation is unlikely to reveal the extent of the immune response since it by-passes the natural route of infection and involves a form of the fungus which is not normally present in the blood of infected insects. It is to be expected that signal exchange between insect and fungus during penetration of the cuticle plays a significant role in the orchestration of the blood borne defence immune responses. Therefore, the object of the present work was a description of the immune responses of the desert locust, *S. gregaria*, to topically applied conidia of isolates of *Metarhizium* spp.

2.2 Materials and Methods.

2.2.1. Chemicals.

All chemicals were analytical grade and purchased, unless otherwise stated, from Sigma or BDH.

2.2.2. Insect cultures

Adult males of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae), 14 days after final ecdysis were used in all experiments unless otherwise stated. Locusts were maintained in metal cages in a controlled environment insectary at 31±4°C, 25-30% RH under a photoperiod of 16 h day, 8 h dark. The locusts were fed daily with fresh wheat shoot, bran (supplemented with dried brewer's yeast) and water. The latter two were supplemented with 5% of an antiprotozoal solution (w/v; 4.26% sodium sulfamethazine; 3.65% sodium sulfathiazole; 3.13% sodium sulfamerazine; all from Sigma Chemical Co.) to suppress growth of the sporozoan parasite *Malamoeba locustae*.

2.2.3. Culture of fungi.

2.2.3.1 Preparation of conidiospores.

Metarhizium flavoviride (isolate.330189 s.s) was originally isolated from *Ornithacris cavroisi* (Orthoptera: Acrididae, Niger). It was supplied by Dr. Chris Prior, IIBC, Silwood park, Ascot and has been passaged through *S. gregaria* to maintain its pathogenicity.

Cultures of *M. flavoviride.* were harvested from 1/4 strength Sabouraud Dextrose Agar (see Appendix 1) grown at 28°C for 7-10 days under constant light.

Conidia were harvested from sporulating plates by pouring ten ml of cotton seed oil (Sigma) onto each plate and a bent glass rod was used to disrupt the conidiospores from the mycelial mat. The resulting spore suspension was then filtered through sterile muslin to remove any mycelia and centrifuged at 3000 rpm for 5 min. After resuspension in oil, the spore suspensions were then sonicated for 5 min to break up any aggregates of spores. The concentration of spores per ml filtrate was determined using an improved Neubauer haemocytometer.

2.2.3.2. Preparation of blastospores and mycelial fragments.

One ml of a 1 x 10^6 spores/ml suspension of *M. flavoviride* (330189 s.s.) was inoculated in 100 ml of 1/4 strength Saburauds Dextrose broth and incubated in an orbital shaker (120 r.p.m.) at 28°C for 48 h. Blastospores were harvested by filtration through a double layer of muslin. The filtrate was centrifuged at 3000 r.p.m. for 5 min. The pellet was washed once in phosphate buffered saline (PBS; pH 7.5; see appendix 1) in endotoxin-free water. The final concentration of spores was 1 x 10^7 blastospores/ml

To obtain mycelial fragments, 48 h old 1/4 SDA plates of *M. flavoviride* were harvested with a sterile loop and washed in endotoxin-free water before centrifuging at 3000 r.p.m. for 5 min. The pellet was resuspended in 5 ml PBS and homogenised to generate mycelial fragments in the same size range as blastospores. The homogenate was washed twice in PBS and resuspended in PBS to a final concentration of 1 x 10^7 fragments/ml.

2.2.3.3. Preparation of protoplasts.

Protoplasts were prepared using the method of Bernier *et al.* (1989) and supplied by Miss K.M. Das. Briefly, mycelia were obtained by inoculating 250 ml Czapek Dox complete broth (see Appendix 1) with 10^8 conidiospores of *M. flavoviride* and incubating at 27 ± 2°C for 2 days. The hyphae were extracted as above and resuspended in 20 ml treatment solution (25 mM 2-mercaptoethanol, 5 mM EDTA) and incubate at room temperature for 30 min. The mycelia were centrifuged at 3500 rpm for 10 min, washed in 0.6 M KCl and resuspended in 4 ml of 0.6 M KCl. To 1.5 ml of this suspension was added an equal volume of Novozym 234 solution (10mg enzyme per ml 0.6 M KCl). This solution was incubated at 28°C for 3 h in a shaking waterbath. Protoplasts were obtained by centrifuging the digest at 3000 rpm for 5 min at room temperature. The pellet was washed twice in 0.6 M KCl and adjusted to a final concentration of 10^8 protoplasts/ml.

2.2.3.4. The effect of antiprotozoal drugs on the germination of *M. flavoviride* (isolate 330189).

Conidiospores of *M. flavoviride* were harvested in sterile distilled water direct from 1/4 strength SDA plates (section 2.2.2.1). The concentration of spores was adjusted to 3 x 10^8/ml. One hundred µl aliquots of spore suspension were placed in 7cm Petri plates and 5ml of 0.05% yeast extract medium containing differing amounts of anti-protozoal drug (final concentrations of (w/v) 1, 2, 4, 5, 8, 10, 25 %) was added. Plates were incubated at 28°C for 16h under constant light. The yeast extract medium was poured off and the Petri plate observed under a phase contrast microscope. Conidia that exhibited germ tubes were scored

as positive. Results were expressed as a percentage of the total number of conidia counted. Two hundred conidia were counted per Petri plate and there were 4 replicates per treatment. The drugs had no effect on germination after 16 h at concentrations up to 25% (w/v) of drug in the medium.

2.2.4. Glassware.

All glassware for haemolymph studies was rinsed with acetone followed by concentrated dimethyldichlorosilane (BDH) and left to dry overnight in a fume cupboard (silanisation). To remove any endotoxin, glassware was then rinsed for 2 min in 1 % (w/v) E-Toxa clean (Sigma) and baked for 2.5 h at 180°C

2.2.5. Treatment of *S. gregaria*.

Locusts were chilled at 4°C for 1 h prior to topical inoculation with 5 µl (1.5×10^7 spores per ml) of a conidiospore suspension. This was the LD_{50} dose detailed by Bateman *et al.* (1993) and was applied under the pronotum via a Hamilton syringe (Hamilton Corp., Reno, Nevada). Control locusts were applied with cotton seed oil alone. Insects were housed individually in an environmental incubator at 28±2°C with a 16h light: 8h dark photoperiod and were fed 5 blades of wheat daily. The wheat was not treated with anti-protozoal drugs in order to negate any possible pharmacological effect that they may have on the host immune system.

2.2.6. Collection and treatment of haemolymph.

2.2.6.1. Enzyme assays and total haemocyte counts.

Haemolymph was collected from the arthrodial membrane of the hindleg of locusts. The membrane was first swabbed with 70 % ethanol, allowed to air dry and then pierced with a sterile needle. The haemolymph was collected using a 10 µl Eppendorff Pipetman over ice to prevent coagulation.

For protein determinations and enzyme assays, a known volume of haemolymph was diluted 40x with sterile ice cold anticoagulant buffer (Appendix 1). The diluted haemolymph was then centrifuged at 13000 rpm for 10 min at 4°C and the decanted supernatant stored at -20°C until used. To ensure that the plasma contained no cellular debris, it was checked for phase bright material under a phase contrast microscope.

To perform the total haemocyte count (THC), haemolymph was collected in 100µl glass capillary tubes and transferred to an improved Neubauer haemocytometer. The number of cells in 5 small squares was counted and the THC calculated.

2.2.6.2. Differential haemocyte counts.

To prepare haemolymph for the counting of individual sub-populations within the whole, haemolymph was collected by bleeding a drop of blood onto a meniscus of Hoyles saline (Appendix 1) which was resting on the tip of a 1ml syringe containing 400 µl of saline. The blood was then withdrawn into the syringe and allowed to mix with the saline. The diluted haemolymph was then expelled onto E-Toxa treated glass

microscope slides. The slides were then incubated in a moist chamber for 10 min at room temperature to allow the blood cells to adhere to the slide. The haemocytes were observed under phase contrast at 400x magnification using an Olympus light microscope linked to a photographic system.

2.2.6.3. Nodule counting

To count nodules circulating in the haemolymph, individual locusts were chilled on ice for 4 min prior to injection of 400 µl ice cold anticoagulant buffer into the 3rd segment of the dorso-ventral abdomen. The insects were then left for a further 3 min on ice prior to making an incision in the neck membrane. Ice cold anticoagulant buffer (400 µl) was then flushed into this aperture and the liquid that emerged was collected in a cell counting chamber (Graticules Ltd). Nodules were counted and measured in 100 squares (this is equivalent to 50 µl) using an eyepiece graticule. A nodule was considered as being an aggregate of 10 or more cells. Results were expressed as number of nodules per ml diluted haemolymph

2.2.7. Determination of Blood Volume.

To determine the blood volume of locusts infected with *M. flavoviride*, 25 µl of 2% (w/v) amaranth in Locust Ringer's saline (Appendix 1) was injected into the body cavity of the insect. This was done by injecting the amaranth using a Hamilton syringe into the 3rd abdominal segment in an anterior direction. Samples of blood were removed every 10 min for 50 min after puncturing the arthrodial membrane with a needle. Five µl of haemolymph was collected using microcap (Camlab) capillary tubes. The 5 µl samples were then placed in

1 ml Ringer's saline in plastic microcuvettes and inverted to ensure mixing. The optical density of the solution was determined using a Pye Unicam PU8650 visible spectrophotometer (Philips) at 520nm. A calibration curve was established using known amounts of amaranth. A graph was drawn of amaranth concentration versus time. The line was extrapolated back to zero and the blood volume determined from the extent of dilution of the amaranth at the time of injection. The rate of excretion of amaranth was determined from the slope of the line.

2.2.8 Protein Determination.

The protein concentration of solutions in mg/ml was determined using the Bradford assay (Bradford, 1976) which was supplied in kit form (Bio-Rad).

2.2.9. Enzyme Assays

2.2.9.1. Phenoloxidase assay.

Phenoloxidase (PO) activity was monitored spectrophotometrically as the formation of dopachrome (Horowitz and Shen, 1952). Aliquots of 100 µl of diluted haemolymph plasma (see 2.2.6.1) were incubated with L-Dopa (L-β-3,4-dihydroxyphenylalanine) (100 µl; 3mg/ml in sodium cacodylate buffer, 10mM; pH 6.0) for 5 min at 30°C. The mixture was made up to 1 ml with cacodylate buffer before measuring absorbance at 490 nm using a Pye Unicam PU8650 visible spectrophotometer (Philips). The specific activity of phenoloxidase was expressed as units of A_{490}/min per mg of protein. One unit of activity was defined as being the amount of enzyme that increases the absorbance

reading at a rate of 0.001 absorbance units per min. It should be noted that this procedure may have resulted in suboptimal enzyme assay conditions.

To determine the amount of prophenoloxidase present in haemolymph samples, the above assay was performed with a slight modification. One hundred µl of the diluted haemolymph sample (see 2.2.6.1) was incubated with 100 µl of the serine protease, chymotrypsin (1 mg/ml in sodium cacodylate buffer) for 30 min prior to the addition of L-Dopa. Then 100 µl of the pre-incubated mixture was assayed as above.

2.2.9.2. Lysozyme activity.

A modification of the method of Powning and Davidson (1973) was employed. The standard reaction medium contained 0.5 mg/ml of dried *Micrococcus lysodeikticus* cell walls in 10 mM sodium phosphate buffer (pH 5.5). Twenty µl of the diluted haemolymph (see 2.2.6.1) was added to 1.2 ml of the *M. lysodeikticus* suspension and the mixture was left to stand for 30 min at 30°C. 0.5 ml of 0.5 M sodium Na_2CO_3 was then added before reading the turbidity of the suspension in a spectrophotometer at 450nm. The initial absorbance readings under these conditions were between 1.5 and 1.6 when measured against 10 mM phosphate buffer. One unit of lysozyme was defined as the amount of enzyme that decreases the absorbance reading at a rate of 0.001 absorbance units per min. the specific activity of the lysozyme in the samples is defined as units of A_{450}/min per mg protein.

2.2.9.3. Acid Phosphatase Assay.

A modification of the spectrophotometric method of Anderson *et al.* (1992) which utilises the substrate p-nitrophenol phosphate was used.

Nine hundred µl of 0.1M sodium citrate (pH 4.8) was added to 500 µl of 12mM p-Nitrophenol phosphate (Sigma) in a test tube. The assay was initiated by the addition of 100 µl of diluted haemolymph (see 2.2.6.1). Control tubes consisted of the reaction mixture with 100 µl of distilled water. The tubes were incubated in a 37°C water bath for 2 h. After this period, the reaction was stopped and the colour developed by the addition of 10ml 0.05M NaOH. Aliquots of the samples were then analysed in a spectrophotometer at 410nm. A calibration curve was generated using commercial acid phosphatase. One unit of activity was defined as being the amount of enzyme that increases the absorbance reading at a rate of 0.001 absorbance units per min.

2.2.10. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Polyacrylamide slab gels measuring 200 x 200 x 2 mm were cast from a mixture containing 15 ml stock acrylamide (Protogel, National Diagnostics, Manville, New Jersey); 12 ml separating gel buffer (Appendix 5), 3 ml distilled water, 0.5 ml ammonium persulphate and 0.05 ml N,N,N',N', Tetramethylethylenediamine (TEMED). When polymerization was complete, a stacking gel with castellated wells was cast on top of the resolving gel from a mixture containing 0.4 ml stock acrylamide, 1.2 ml stacking gel buffer (Appendix 5), 0.9 ml distilled water, 0.25 ml ammonium persulphate and 0.005 ml TEMED.

To prepare samples for electrophoresis, samples of diluted haemolymph fractions were lyophilised in a Savant Speedvac concentrator (Savant, New York). The amount of sample containing 40 µg protein was determined. The sample was resuspended in 50 µl sample buffer (Appendix 5) and boiled for 3 min. Fifty µl samples were loaded onto the

gel which was immersed in electrophoresis buffer (Appendix 5). Electrophoresis was performed at 4°C at a constant voltage of 200V for 30 min. After this, the voltage was increased to 350V for 3 hours or until the tracking dye had reached the bottom of the resolving gel.

Staining of the gels was done by incubating gels in Coomassie blue stain (20% methanol; 10% acetic acid; 0.1% Coomassie brilliant blue R-250) overnight at room temperature. protein bands were visualised by putting the gels in 3 washes of destain (25% methanol; 10% acetic acid) over 48 h or until the bands were visible.

2.2.11. Densitometry.

Densitometry of polyacrylamide gels was performed on a 2202 Ultrascan laser densitometer (LKB) linked to a C1-10 integrator (Milton Roy).

2.2.12. Monolayer Preparation.

In order to obtain monolayers, locusts were chilled on ice for 10 min to make handling easier. The arthrodial membrane of the hindleg was treated as in section 2.2.5 and the blood collected onto a meniscus of Hoyle's saline on a syringe containing 200 µl of chilled saline. The syringe was inverted several times to disperse the cells before dispensing 50 µl onto endotoxin-free coverslips (see 2.2.4). Two coverslips were placed onto an endotoxin-free microscope slide and incubated for 15 min at room temperature in a moist chamber in order to allow the haemocytes to fall out of suspension and adhere to the glass. The monolayers were washed four times with Hoyle's saline to remove plasma and any non-adherent material. The slides were observed under a phase contrast microscope to

ensure successful formation of monolayer. Preliminary experiments established that the monolayers were stable for up to 24 hours at room temperature and could be used for a series of experiments.

The monolayers were used to investigate the effects that fungal bodies had on individual haemocyte populations. Monolayers were washed twice in PBS before being overlaid with 100µl samples of mycelial fragments, blastospores or protoplasts. The monolayers were incubated in moist chambers for 1 h. Any unbound material was washed off with PBS and the preparation was viewed under a light microscope. Monolayers were scored for numbers of cells binding fungal bodies. Overlays were also performed using 100µl of 1 µg/ml solutions of laminarin and zymosan. These monolayers were subsequently stained for acid phosphatase activity (see 2.2.12.2).

2.2.12.1. Staining for superoxide radicals (O_2^-) using Nitroblue tetrazolium (NBT) reduction.

The method of Anderson *et al.* (1992) was used to study the generation of superoxide radicals (O_2^-) by haemocytes. Monolayers were washed in PBS (pH 7.5) to displace the Hoyle's saline. This was because the Hoyle's saline was sufficiently acidic to inhibit the reduction of NBT by O_2^-. Monolayers were overlaid with 50 µl PBS followed by 50 µl of a 2mg/ml solution of NBT in PBS. This provided the correct osmotic environment for the monolayers. The monolayers were incubated in a moist chamber for 2 h at room temperature and washed with PBS. The cells were then observed as wetmounts using a bright field microscope. The presence of O_2^-. was indicated by the presence of dark blue deposits of formazan (the reduced form of NBT).

The specific reduction of NBT by superoxide radicals is indicated when the reduction is inhibited by the enzyme, Superoxide dismutase (SOD). Monolayers were set up as described in section 2.2.12 and overlaid with a stimulating agent in PBS containing 50 ng/ml SOD. The monolayers were incubated for 15 min prior to the addition of 50 µl NBT solution. They were then incubated at room temperature for 1 h. NBT reduction using stimulated and unstimulated haemocyte monolayers was carried out with and without SOD to demonstrate any reduction specificity.

2.2.12.2. Staining for Acid phosphatase activity.

Monolayers were stained for acid phosphatase activity using a Sigma diagnostic kit designed for lymphocyte enzymes. The assay utilised the simultaneous capture principle to generate an insoluble chromogenic Naphthol AS-BI-GBC complex which is dark red in colour.

The staining procedure was carried out according to the manual provided. Monolayers of more than 100 cells were stained and cells that were dark red in colour scored as positive for acid phosphatase.

2.2.13. Statistical analysis.

Statistical analysis was performed on all data. Significant differences were determined using Students *t* test.

2.3. Results.

2.3.1. Symptoms of disease.

Infection of *S. gregaria* with the green muscardine fungus, *M. flavoviride* brings about mycosis and ultimately death of the host with the subsequent emergence of the fungus from the cadaver (Figure 2). *M. flavoviride* (isolate 330189 s.s.) has a median lethal time (MLT) of 4.41 days as opposed to *M. anisopliae* (isolate ME1) which has a MLT of 7.34 days (C. Prior, Pers. Comm.; see Appendix) for a dose of 7.5 x 10^4 spores.

Preliminary experiments showed that, after inoculation of insects with *M. flavoviride* (330189) there was a progressive decline in appetite confirming the observations of Moore *et al.* (1992) and Seyoum *et al.*, (1994). An inoculum of 7.5 x 10^4 spores caused the first deaths after 4 days. Dead locusts were pink in colour (figure 3) and in some cases melanised cuticle was observed.

An initial survey experiment was carried out. This involved inoculating groups of locusts with 7.5 x 10^4 spores of one of 15 *Metarhizium* isolates available. Symptoms induced by infection were noted as well as the number of haemocytes and the extent of fungal growth in the blood 4 days after inoculation (Table 3). There was variation in symptom expression within each experimental group. There was no apparent relationship between the extent of melanisation or occurrence of paralysis of infected insects and isolate virulence. Not all isolates colonised the blood over the time scale of the experiment. Haemocyte count varied between isolates. In some cases it was not significantly different from the controls while in others it was significantly greater or lower. Similar variability occurred with weight changes in experimental animals, reflecting

Figure 2 Cadaver of *S. gregaria* after infection with the green muscardine fungus, *M. flavoviride* (330189). Death occurred 4 days post inoculation. The cadaver was incubated in a moist chamber for 4-5 days until the fungus emerged and sporulated on the surface.

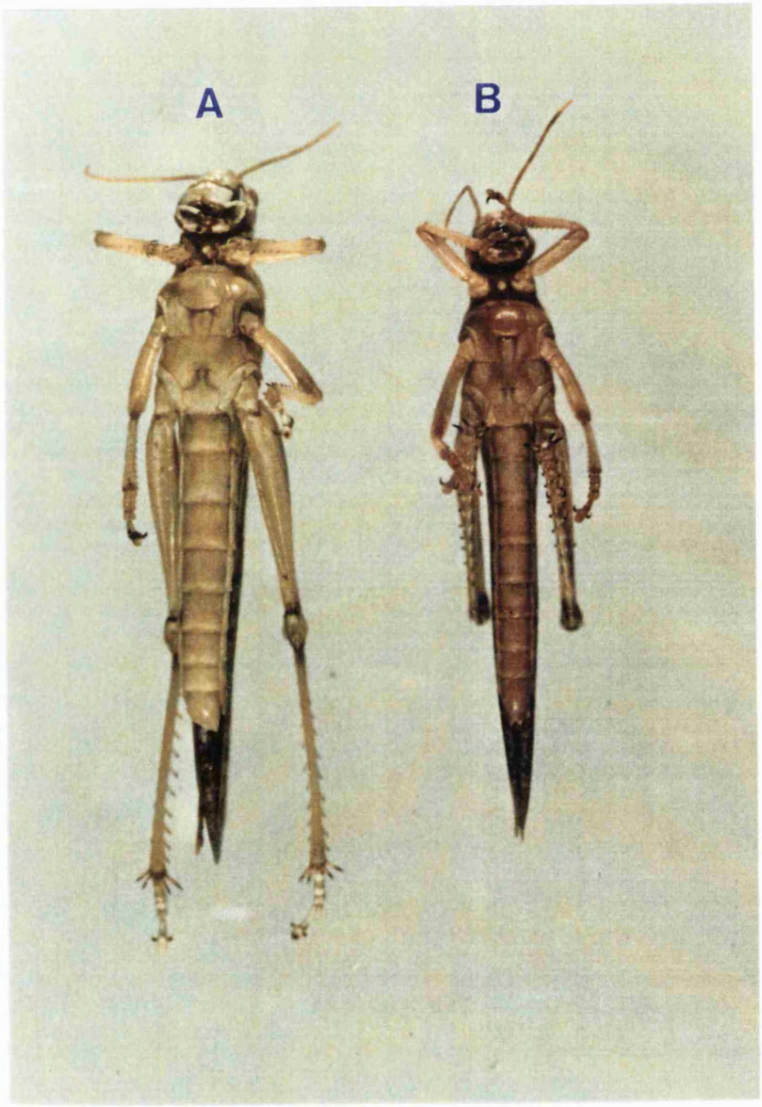


Figure 3. Cadaver of (A) control *S. gregaria* showing normal colouration and (B) *S. gregaria* after inoculation with *M. flavoviride* (330189). Death occurred 4 days post inoculation.

Table 3. Survey of the response of adult male desert locusts to infection with isolates of *M. aniosopliae* and *M. flavoviride*.

Strain	Mean % Weight gain or loss	Paralysis[a]	Melanisation[a]	Total haemocyte count (x 10^6/µl) mean ± S.E.	Total fungal count (x 10^6/µl)[b] mean ± S.E.
Control	+5.43	0	0	3.01 ± 0.31	0
ARSEF 324[c]	+10.24	1	4	2.25 ± 0.19	10.00 ± 1.86
330189[c]	-6.54	1	0	1.54 ± 0.23	47.00 ± 10.47
324673[c]	-6.24	0	0	3.59 ± 0.57	11.67 ± 3.69
ME1[d]	-3.43	0	0	7.25 ± 1.63.	0
168777ii[d]	+6.13	0	1	0.81 ± 0.10	38.36 ± 1.93
299984[d]	+0.10	0	0	2.57 ± 0.31	0
ARSEF 2023[c]	+9.93	0	4	6.55 ± 0.97	0
152222[d]	+2.98	2	0	1.25 ± 0.30	59.00 ± 15.34
299981[d]	+1.36	0	1	3.75 ± 0.53	30.20 ± 1.58
298061[d]	+18.98	0	1	0.83 ± 0.09	48.40 ± 12.78
298059[d]	+4.81	0	0	2.75 ± 0.93	78.00 ± 16.39
ARSEF 727ii[d]	-6.80	0	0	1.81 ± 0.17	0
ARSEF 438[d]	+16.27	0	3	1.87 ± 0.12	0
ARSEF 439[d]	+2.81	0	2	3.94 ± 0.39	0
ARSEF 440[d]	+15.21	1	1	3.21 ± 0.34	0

[a] Figures represent the number of insects (out of a total of 4) exhibiting melanisation or paralysis.
[b] Data taken for insects that showed a trend of infection or not. If a single insect out of 4 was (or was not) infected, the data point was ignored.
[c] Denotes *M. flavoviride* isolate. Isolates are presented in order of median lethal time (MLT) with the first isolate having the quickest MLT (for data see section 3.3).
[d] Denotes *M. anisopliae* isolate.

differential effects on feeding. There were no obvious relationship between any of these parameters and isolate virulence. Nor was there any correlation between the haemocyte count and the fungal count.

2.3.2 Identification of blood cells in *S. gregaria*.

The main types of haemocyte were identified in the blood of the desert locust (figure 4). These were termed vacuolated plasmatocytes (vPLs) which spread on E-toxa-coated glass and contained large numbers of granules and vacuoles; avacuolated plasmatocytes (aPLs) which spread on E-toxa-coated glass slides and contained granules whilst vacuoles were absent; coagulocytes (COs) which lysed or degranulated *in vitro* and may appear as either as a nucleus and remnants of surrounding cytoplasm attached to the slide by thin streamers of diffuse coagulum; granular cells (GRs) which were phase bright, granule containing round cells anchored by thin filopodia. The presence of small round phase bright cells which are prohaemocytes was also observed infrequently. These haemocyte types were essentially similar to those described by Lackie *et al.* (1985) except that the PL sub-population appeared to be divided into heavy vacuolated PLs and avacuolated PLs.

2.3.3. Progression of Infection of *M. flavoviride* in *S. gregaria*.

Fungal bodies were observed routinely in the blood of locusts 3 days after inoculation with *M. flavoviride* (figure 5). However, transfusion of uninoculated insects with 10 µl of blood taken from 2 or 3 day inoculated locusts caused mycosis of *M. flavoviride*, suggesting that low levels of the fungus reached the blood within 48 h. Day one blood was not

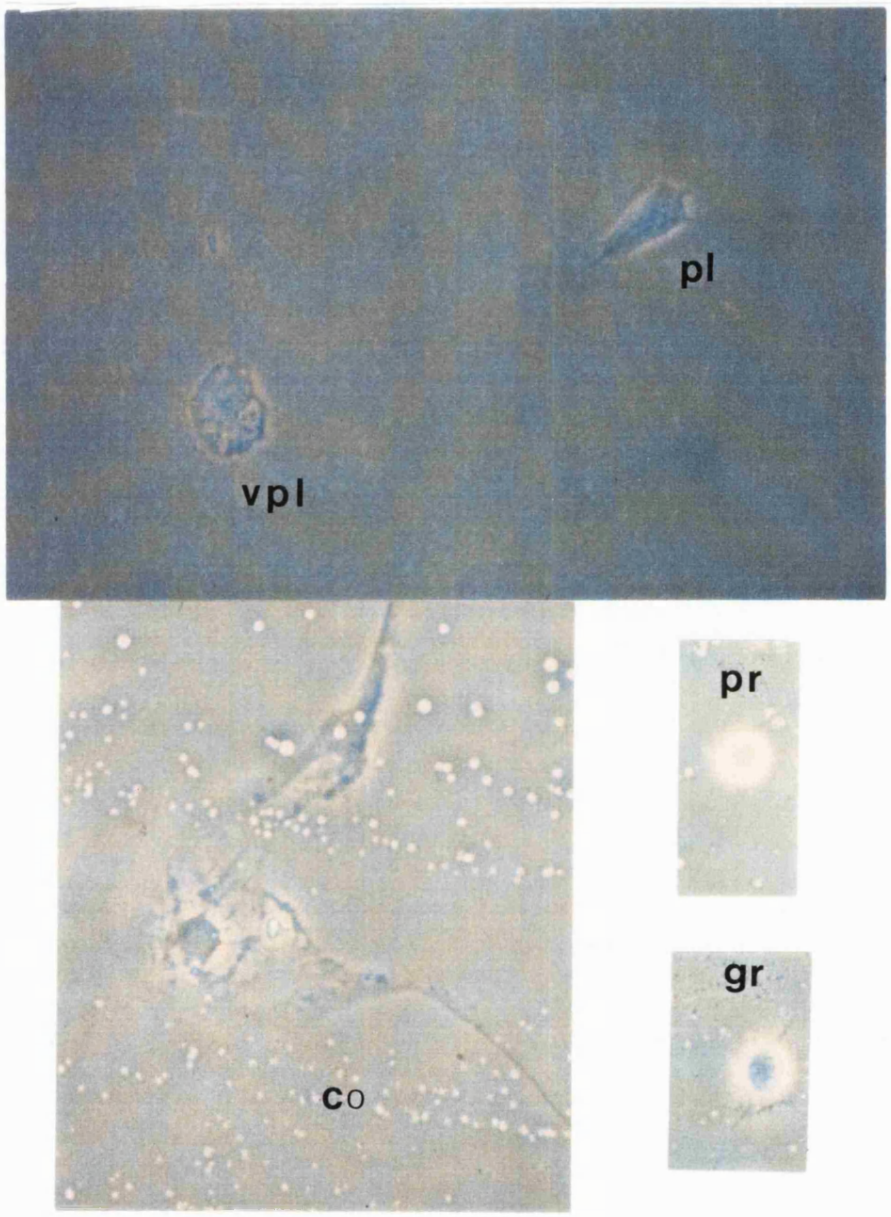
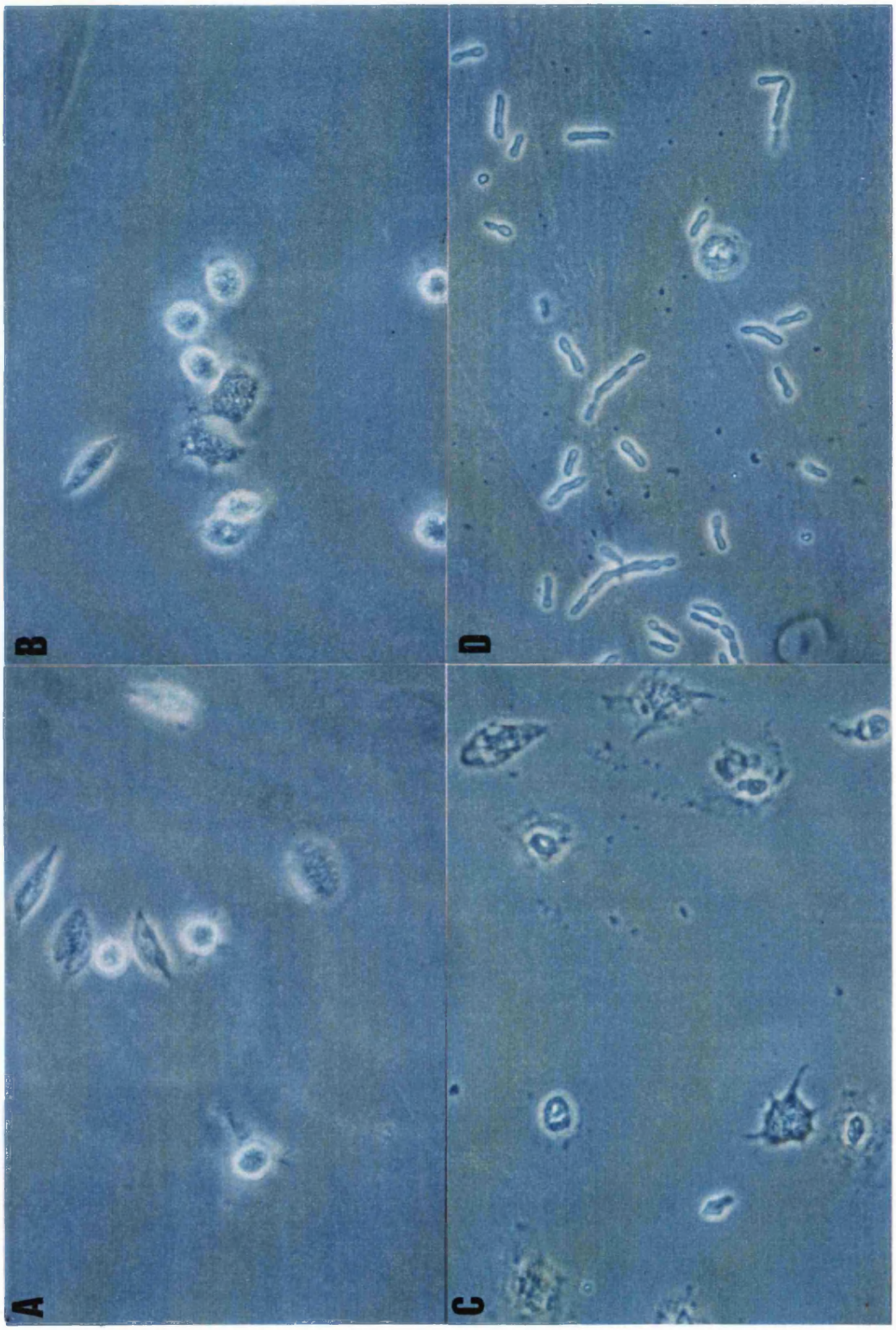


Figure 4. Identification of blood cells from *S. gregaria.* vpl-vacuolated plasmatocyte; pl-avacuolated plasmatocyte; co-coagulocyte; gr-granular cell; pr-prohaemocyte. (Magnification x400).

Figure 5 (overleaf). Time course of infection of *M. flavoviride* (isolate 330189) in *S. gregaria.* A; control (0 day): B; 1 day after inoculation: C; 2 days after inoculation: D; 3 days after inoculation. (Magnification x400).


B
D
A
C

infective (data not shown). When locusts were infected with a larger inoculum (5µl of 10^8 spores/ml instead of 5µl of 10^7 spores/ml) small numbers of round, phase bright objects ca. size of granular cells appeared in the blood 48h after inoculation (figure 6). These bodies were similar in appearance to protoplasts produced *in vitro* from *M. flavoviride* mycelium (compare fig. 6 with fig. 15). If fungal in origin, this would account for the infectivity of 48h blood.

The presence of antiprotozoal compounds in the locust diet had no effect on the germination of *M. flavoviride in vitro* (see Appendix 2). Blood was taken from the insects at 24 h intervals during infection and the total haemocyte count (THC) recorded (table 4). The cell count after 2 days was significantly higher than control counts but declined over the subsequent 48 h to values significantly less than control values. The blood volume of *S. gregaria* did not change significantly during infection (table 5; nor incidentally did the secretion rate from the Malpighian tubules) Therefore, the THC per µl (table 4) is a true representation of the changes in blood cell count during infection. To determine whether changes in THC represented a decline in the whole population or within distinct sub-populations, individual cell types were counted over the course of infection. This differential haemocyte count (DHC) is shown in figures 7 and 8. The contribution of PLs to the THC decreased over the course of the experiment. More specifically, the percentage of vacuolated PLs decreased significantly and were completely absent by day 3. The number of avacuolated PLs decreased as well but were still present on day 4. The proportion of GRs in the blood remained reasonably constant until day 4 when it decreased significantly. Conversely, the proportion of CSs increased significantly over the course of infection to represent over 80% of the total blood cell population.

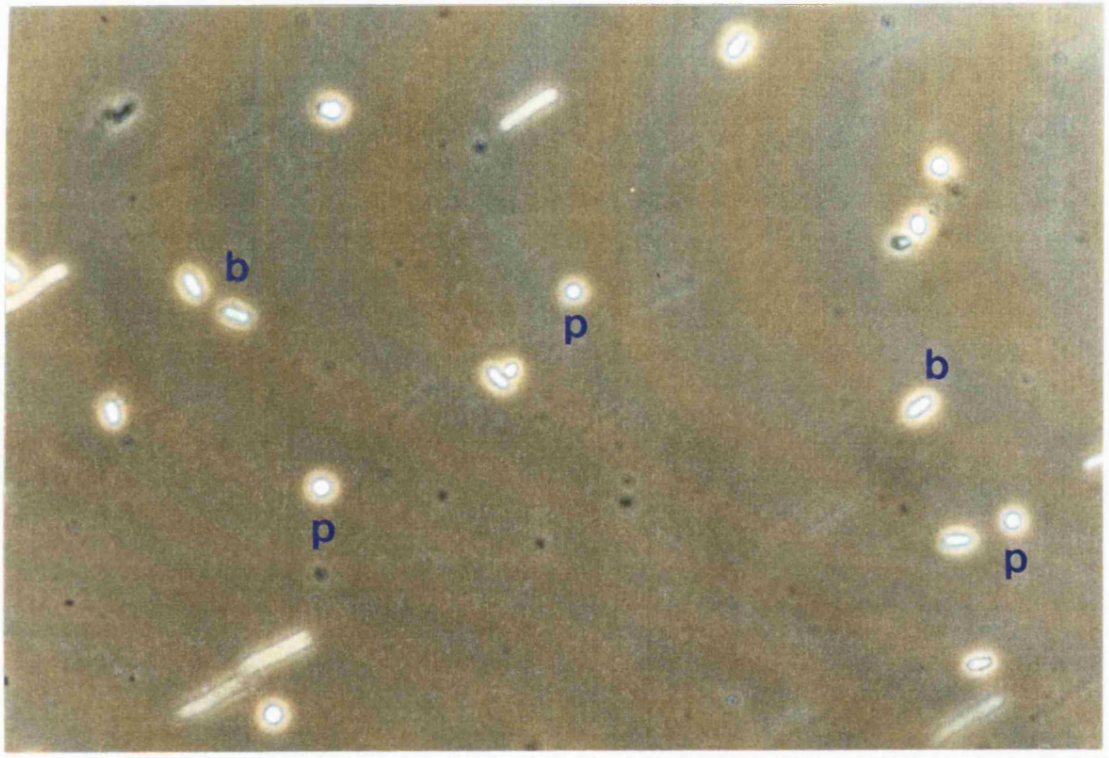


Figure 6. Infection of *M. flavoviride* (isolate 330189) in *S. gregaria* with 7.5 x 10^5 conidispores. Photograph taken 2 days after inoculation. Protoplast-like bodies (P) were observed in the blood. (Magnification x400).

Table 4. Total haemocyte count in adult male *S. gregaria* after topical infection with 7.5 x 10^4 spores of *M. flavoviride* (330189 s.s.)[a].

Days after inoculation[b]	Total haemocyte count (x 10^6; per µl).	
	control	experimental
1	1.85 ± 0.22	2.11 ± 0.24
2	1.67 ± 0.16	2.52 ± 0.18[x]
3	1.27 ± 0.65	1.57 ± 0.21
4	1.50 ± 0.17	0.30 ± 0.07[x,y]

[a] Figures reported with standard error; n=12

[b] Controls were inoculated with cottonseed oil and maintained under the same conditions as infected locusts.

[x] Significantly different from corresponding control value ($p<0.05$).

[y] Significantly different from value on day 1 ($p<0.05$).

Table 5. The effect of topical infection with 7.5 x 10^4 spores of *M. flavoviride* (330189 s.s.).on the (a) blood volumes and (b) rate of secretion of amaranth from Malpighian tubules in adult male *S. gregaria*[a].

Day after inoculation[b]	Blood volume (µl)		Rate of secretion (ng/min)	
	control	experimental	control	experimental
1	212.40 ± 21.94	196.83 ± 9.60	4.72 ± 0.49	4.60 ± 0.50
2	271.43 ± 14.57	210.06 ± 12.45	4.60 ± 1.07	4.01 ± 0.60
3	241.57 ± 26.91	218.30 ± 15.20	4.06 ± 0.29	4.81 ± 0.62
4	202.69 ± 20.62	214.59 ± 13.79	3.49 ± 0.08	3.96 ± 0.62

[a]figures reported with standard error: n=12
[b]day 0 represents controls that were inoculated with cottonseed oil and maintained under the same conditions as infected locusts.

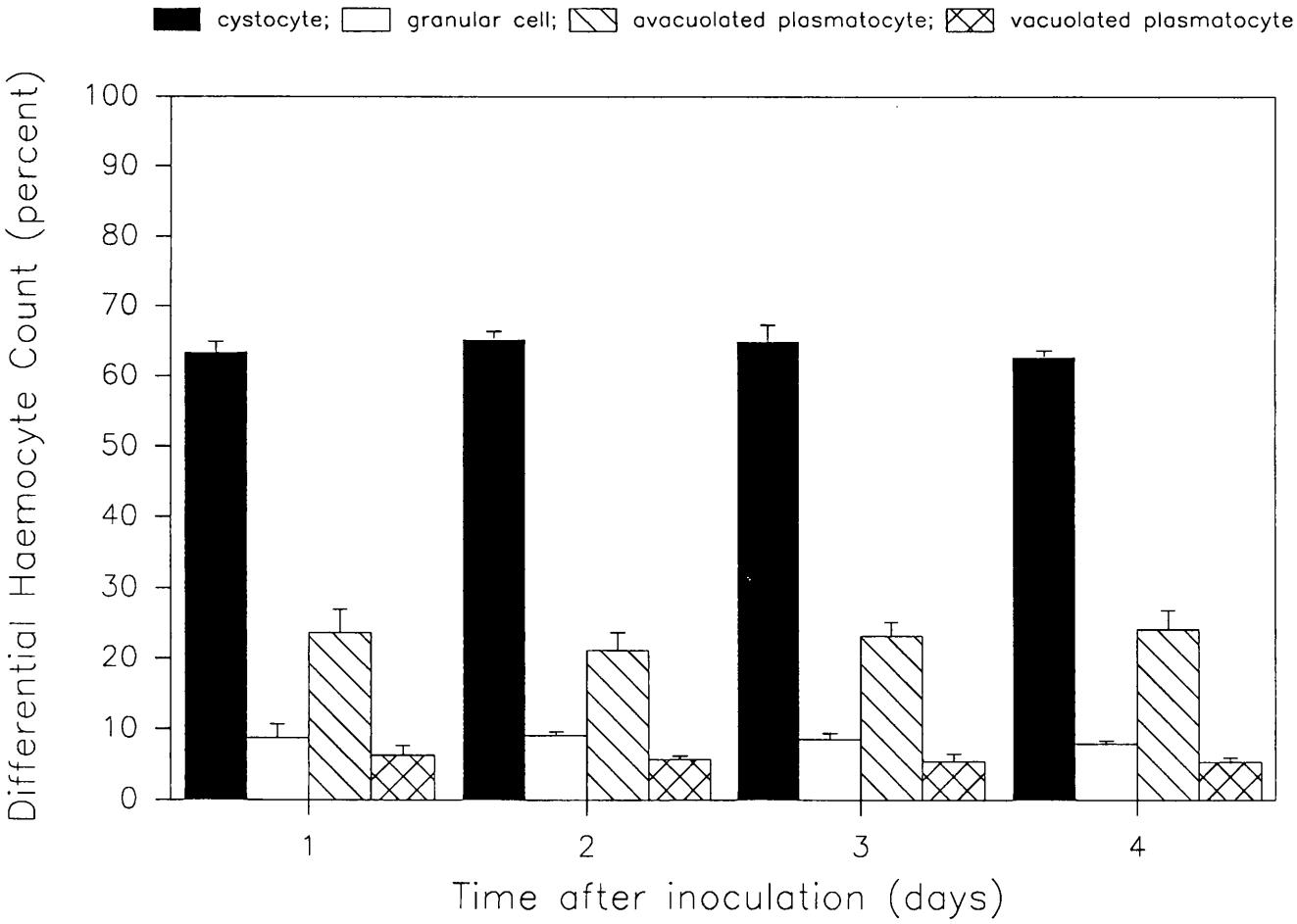


Figure 7: The effect of topical application of cottonseed oil on the differential haemocyte count of *S. gregaria*. S.E. bars are indicated (n=12). These insects served as controls.

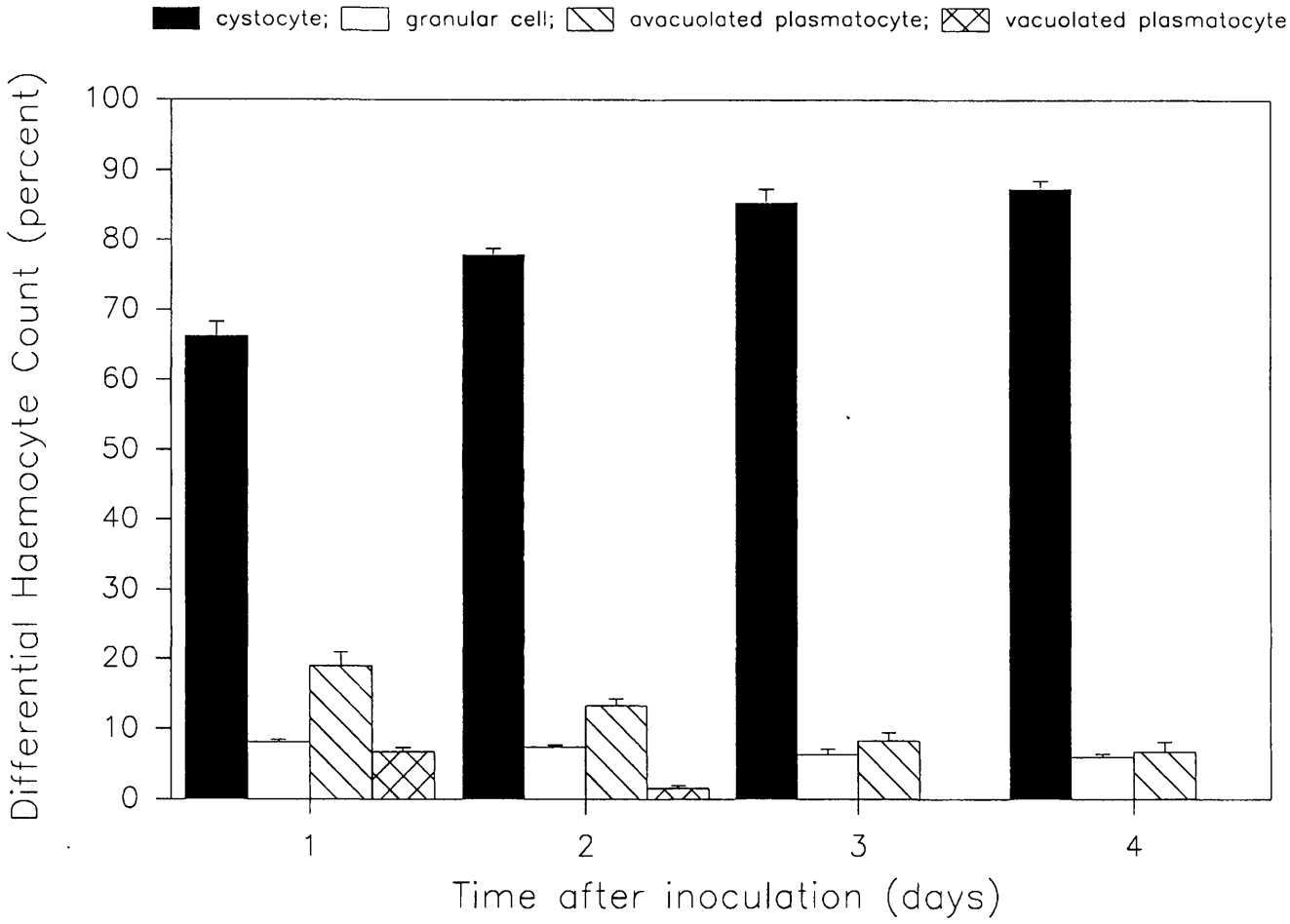


Figure 8: The effect of topical infection with *M. flavoviride* (isolate 330189) on the differential haemocyte count of *S. gregaria*. S.E. bars are indicated (n=12)

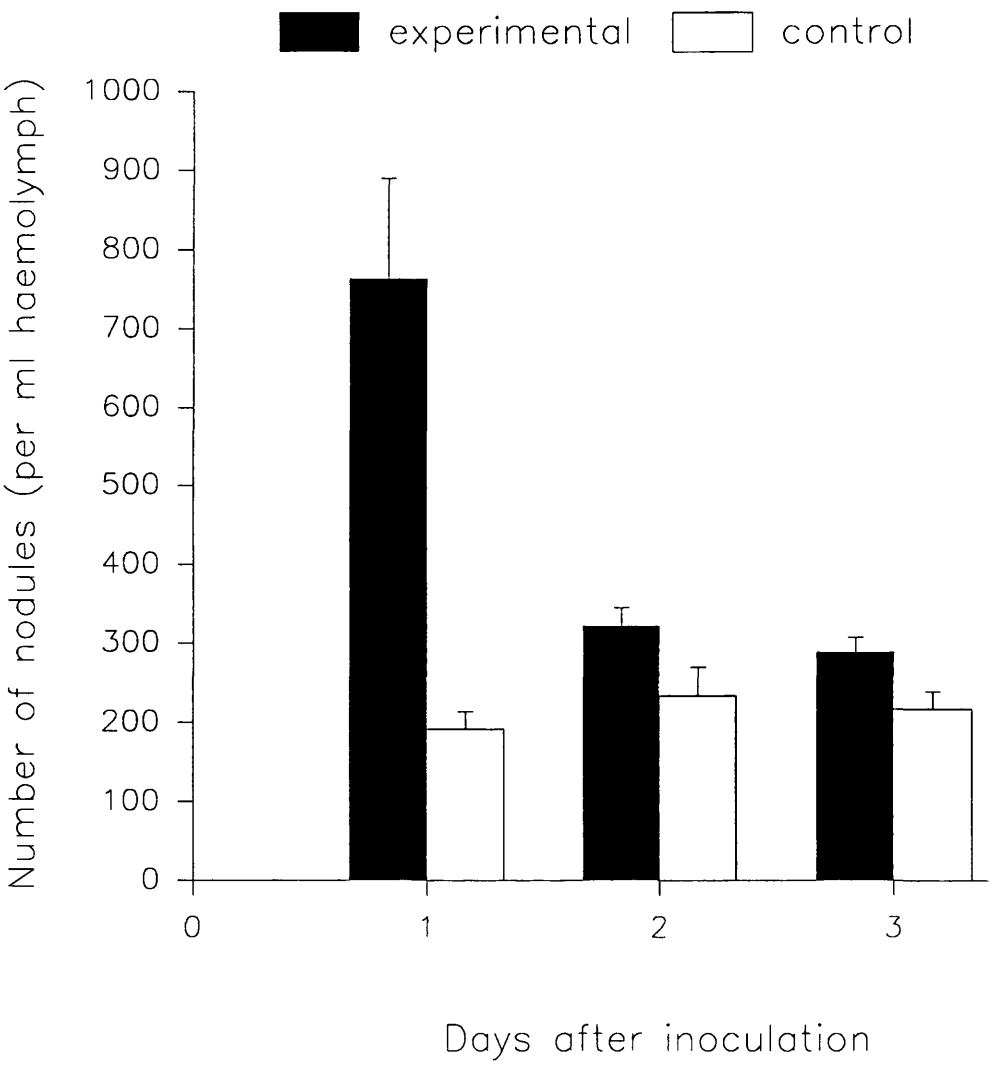


Figure 9: The number of nodules per ml haemolymph from *S. gregaria* after infection with *M. flavoviride* (isolate 330189). S.E. bars are indicated (n=12)

The decline in blood cell counts in the later stages of infection could be a result of freely circulating haemocytes becoming involved in the formation of nodules. Figure 9 shows the change in the number of nodules over the course of infection. Nodules were observed in the blood of control insects (400 per ml haemolymph). This value increased significantly to 775 per ml one day after infection ($p<0.001$). This high value declined but infected insects still had significantly more nodules than controls on day 3.

According to Gunnarsson (1986), the size of nodules induced in *S. gregaria* is dependent upon the nature and size of the inducer. In the present study, a comparison of the distribution of nodule sizes was made (figure 10). It was found that in uninfected controls, nodules had a median size of 55 μm^2. One day after inoculation, the median size of nodules was 112 μm^2 and this value was 77 μm^2 on day 2 and 130 μm^2 on day 3. Statistical analysis of these data (Kruskal Wallis test) indicated , however, that the distributions of nodule sizes in the 4 groups were not significantly different.

2.3.4. Enzyme levels in infected insects

The nodules described above were black in colour, a phenomenon reported many times in the literature. The occurrence of this blackening suggested that melanisation had occurred. This process is caused by the oxidation of phenolic residues on proteins by the enzyme phenoloxidase (PO). An experiment was conducted to see if PO was present in the plasma or haemocytes of *S. gregaria* blood. Table 6 shows that PO was present in greater quantities in the plasma than in the haemocytes. Note, however, thta assay conditions were not the same for plasma and haemocyte lysate

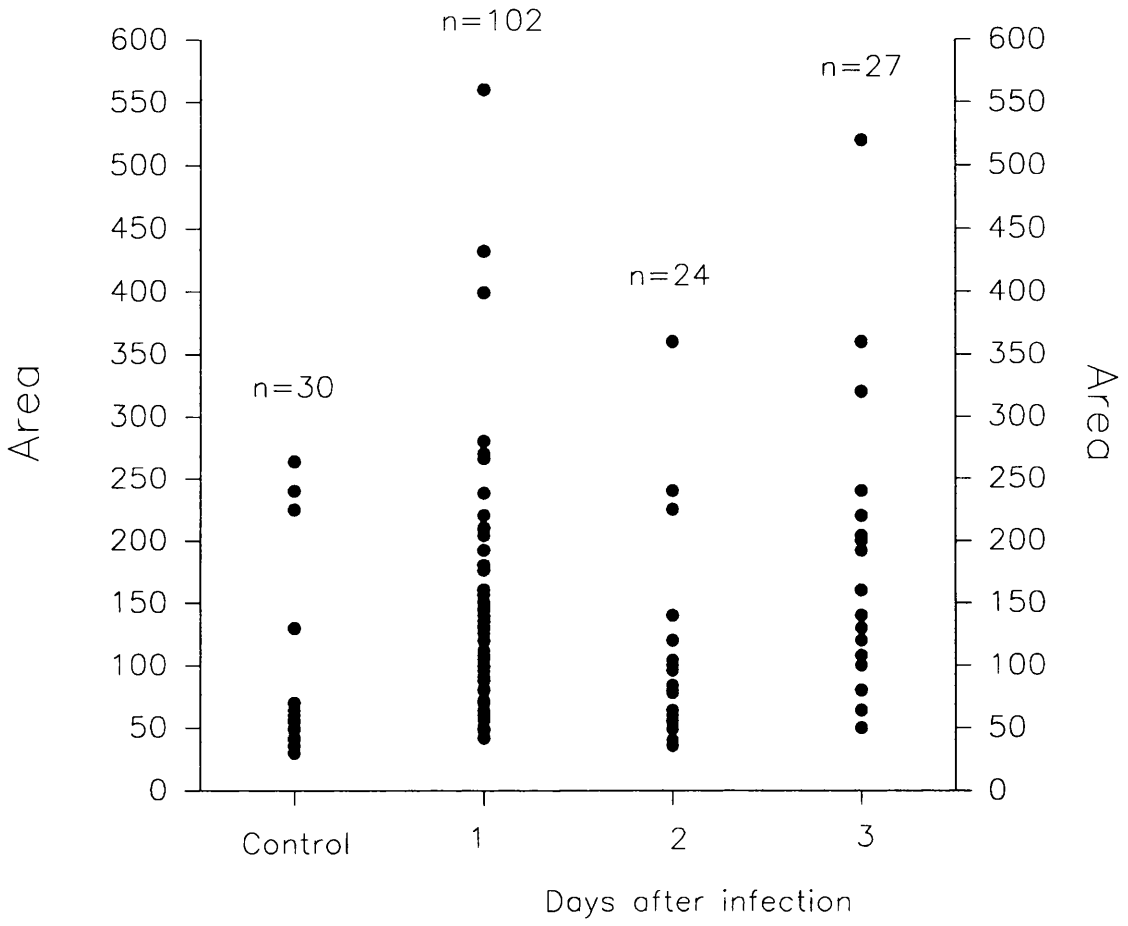


Figure 10: The distribution of size (area) of the nodules observed in the blood of *S. gregaria* after infection with *M. flavoviride* (330189).

Table 6. Prophenoloxidase activation in the plasma and haemocyte lysate supernatant (HLS) from blood of *S. gregaria*.

	Addition[a]	Units of phenoloxidase activity/ml haemolymph ($x10^3$) [b]
Plasma		
	None	1.24 ± 0.15
	Chymotrypsin	149.12 ± 29.57
	Trypsin	47.50 ± 2.73
	Nargase	1.51 ± 0.17
	S. griseus protease	1.11 ± 0.30
	A. oryzae protease	1.49 ± 0.46
	Laminarin	1.37 ± 0.39
Haemocyte Lysate Supernatant (HLS)		
	None	0.24 ± 0.04
	Chymotrypsin	11.47 ± 0.38
	Trypsin	47.50 ± 2.73
	Nargase	0.54 ± 0.07
	S. griseus protease	0.54 ± 0.15
	A. oryzae protease	0.49 ± 0.20
	Laminarin	0.35 ± 0.09

[a] Blood was pre-incubated with exogenous sample for 20 min prior to assay for PO.
[b] n=6; mean presented ± standard error.

preparations. It is possible that this may have led to underestimation of PO in the plasma, which was probably assayed at too low pH values. Upon addition of chymotrypsin, the plasma activity increased 120-fold indicating that PO is present in its inactive form prophenoloxidase (pPO). Another potential activator, trypsin increased the detectable PO activity although to a lesser extent than chymotrypsin. Interestingly, the chymotrypsin increased the haemocyte PO activity by 48-fold whereas the trypsin increased it by nearly 200-fold. Laminarin, an activator of pPO identified previously, failed to activate the proenzyme.

The PO and pPO levels in infected insects were investigated. Table 7 shows that the PO activity decreases during infection. The levels of enzyme in control insects increased significantly between 24 and 48h but declined to levels similar to that for 24h for the rest of the experiment. After 48 h, the level in experimental insects had decreased significantly from control levels and remained at a low level during the course of the experiment. The pattern of pPO activity in infected blood was essentially the converse of the PO, in that it was low 24h after inoculation and increased significantly up to day 4. Prophenoloxidase activity in control blood also increased significantly from 1-2 days but remained at that level until day 4. In control blood, activity was significantly greater than in infected blood on day 1 but significantly less from day 2- day 4.

Another enzyme involved in the immune process is lysozyme, a muramidase that has been reported as antimicrobial. The activity of this enzyme in locust blood plasma decreased significantly by day 2 after infection but recovered a little by the end of the experiment (table 8). Apart from the activity at day 2, none of the data points were significantly different from the controls.

Acid phosphatase was also investigated. Table 9 shows that, 1 day after infection, the levels of plasma acid phosphatase were significantly

Table.7. The effect of topical application of 7.5 x 10^4 conidiospores of *M. flavoviride* on haemolymph plasma protein concentration, phenoloxidase and prophenoloxidase activity in adult male *S. gregaria*[a].

Days after inoculation	Protein concentration (mg/ml haemolymph)		Phenoloxidase Activity (Units/mg protein)		Prophenoloxidase activity (Units/mg protein)[b]	
	control[c]	experimental	control[c]	experimental	control[c]	experimental
1	15.13 ± 0.79	15.42 ± 0.51	496.82 ± 39.25	496.16 ± 58.54	92.40 ± 12.70	30.35 ± 11.33 [x]
2	15.51 ± 1.05	15.71 ± 0.45	764.02 ± 89.98 [y]	209.00 ± 28.17 [x,y]	170.45 ± 43.01	357.49 ± 49.62 [x,y]
3	16.29 ± 1.12	13.50 ± 0.82	528.25 ± 28.74	263.07 ± 47.11 [x,y]	128.63 ± 8.31	414.64 ± 55.37 [x,y]
4	15.23 ± 1.14	12.83 ± 0.56	438.10 ± 20.70	245.99 ± 13.22 [x,y]	148.93 ± 13.48	527.71 ± 81.60 [x,y]

[a]Figures reported plus or minus standard error. n = 12.
[b]Determined by the activation of haemolymphal phenoloxidase with 100µl of a 1mg/ml solution of bovine pancreatric α-chymotrypsin.
[c]Control insects were inoculated with cotton seed oil and maintained in conditions identical to infected insects.
[x] Significantly different from corresponding control value ($p<0.05$).
[y] Significantly different from value on day 1 ($p<0.05$).

Table 8. The effect of topical application of 7.5 x 10^4 conidiospores of *M. flavoviride* on haemolymph plasma lysozyme activity in adult male *S. gregaria*[a].

Day after inoculation[b]	Lysozyme activity (U/mg protein)	
	control	experimental
1	252.57 ± 55.19	128.64 ± 20.27
2	272.63 ± 10.51	85.72 ± 4. 49[x]
3	164.20 ± 18.26	100.62 ± 37.41
4	165.50 ± 22.07	141.94 ± 8.31

[a] Figures reported mean plus or minus standard error. n = 12.
[b] Control insects that were inoculated with cotton seed oil and maintained in conditions identical to infected insects.
[x] Significantly different from corresponding control value ($p<0.05$).

Table 9. The effect of topical application of 7.5 x 10^4 conidiospores of *M. flavoviride* on haemolymph plasma acid phosphatase activity in adult male *S. gregaria*[a].

Day after infection[b]	Acid phosphatase activity (µM pNP/mg protein/min)	
	control	experimental
1	4.30 ± 0.08	11.97 ± 0.24
2	4.69 ± 0.06	6.13 ± 0.16
3	4.28 ± 0.05	7.27 ± 0.33
4	4.43 ± 0.07	6.56 ± 0.16

[a] Figures reported plus or minus standard error. n = 12.
[b] Control insects that were inoculated with cotton seed oil and maintained in conditions identical to infected insects.
[x] Significantly different from corresponding control value ($p<0.05$).
[y] Significantly different from value on day 1 ($p<0.05$).

greater than in controls. This increase was accompanied by a significant decrease in activity by day 2 to a level which was still significantly greater than controls and which was sustained for the rest of the experiment.

2.3.5. Protein changes in infected insects.

Although the concentration of protein in the blood did not vary significantly over the first 2 days of infection, the protein levels in infected blood decreased significantly after 3 days (table 7). The control blood did not differ significantly at any time point from day one blood.

It was possible that individual protein components were fluctuating, reflecting the variation in enzyme activities during mycosis noted above. Figures 11 and 12 show the electrophoretic gels of blood from infected and control locusts. There appear to be fluctuations in the number and size of proteins bands. Analysis of these gels with a densitometer established that this is indeed the case (tables 10 and 11). Proteins can be grouped into one of 3 categories. Those which do not change during the course of the experimental period either in control or infected insects (e.g. 17 kDa., 19.5 kDa.); proteins which are present consistently in controls and decline during infection (e.g. 24.1 kDa., 28.1 kDa.) or proteins which are not present in the controls but appear during infection (e.g. 11.5 kDa., 33.4 kDa., 49.5 kDa.). The most notable proteins are the one of 11.5 kDa which was not found in controls but appeared on days 1and 2 after inoculation and the 33.4 kDa. protein which may be a fungal protein as the time it appears (day 3) is also the time that fungal hyphal bodies are first seen in the haemolymph.

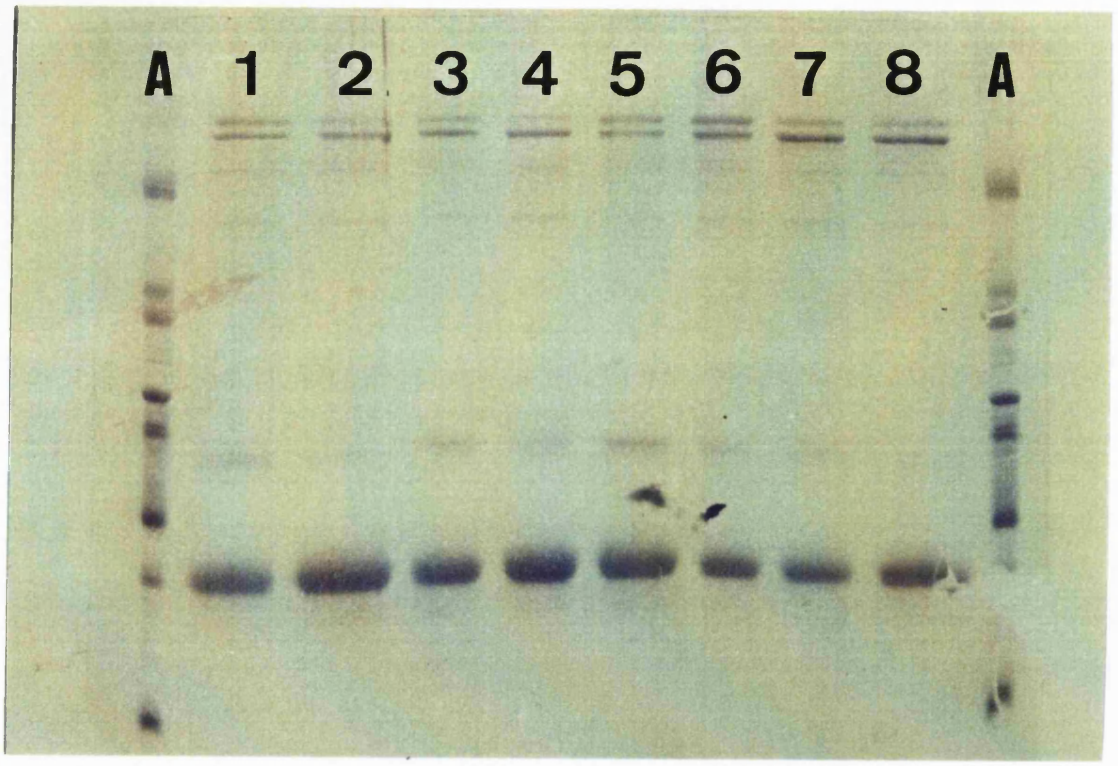


Figure 11 Electrophoretic gel of blood from infected locusts. **Lane 1**; control (day 1): **lanes 2,3,4**; infected blood (day 1): **lane 5**; control (day 2: **lanes 6,7,8**: infected blood (day 2). **Lane A.** SDS-PAGE markers (lactalbumin, 14.2 kDa.; soybean trypsin inhibitor, 21.0 kDa.; trpsinogen, 24.0 kDa., carbonic anhydrase, 29.0 kDa.; glyceraldehyde-3-phosphate dehydrogenase, 36.0 kDa.; egg albumin, 45.0 kDa.; bovine albumin, 66.0 kDa.)

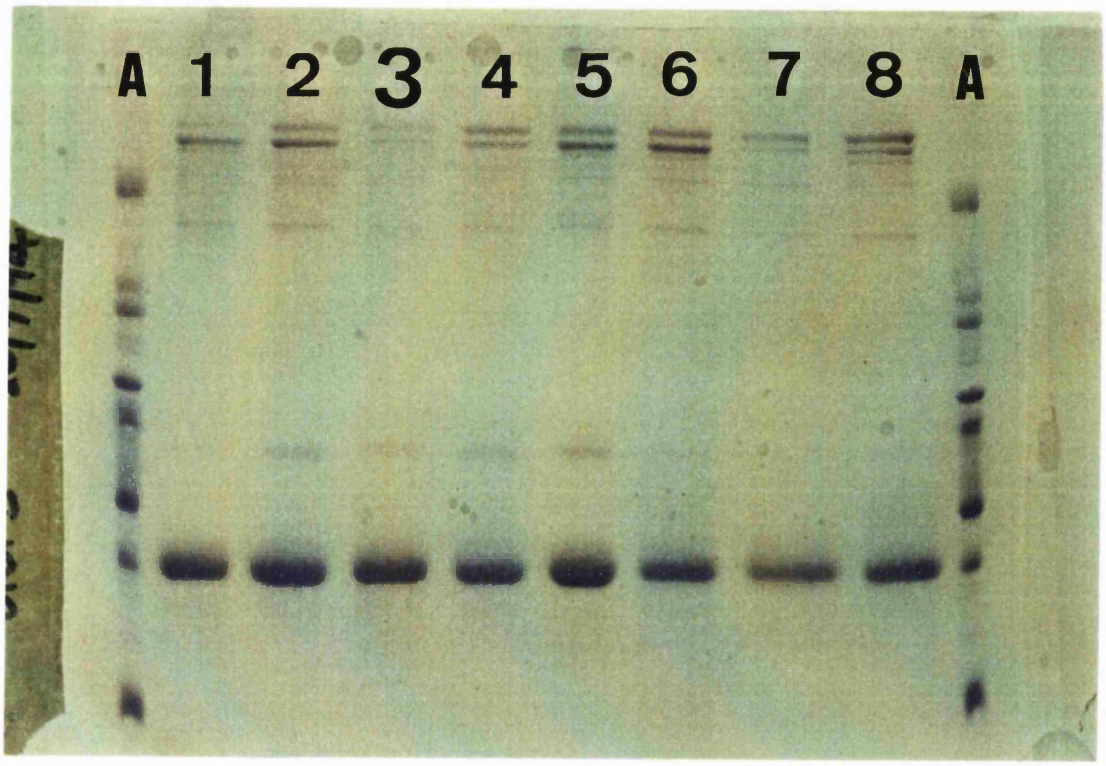


Figure 12 Electrophoretic gel of blood from infected locusts. **Lane 1**; control (day 3: **lanes 2,3,4**; infected blood (day 3): **lane 5**; control (day 4: **lanes 6,7,8**: infected blood (day 4). **Lane A.** SDS-PAGE markers (lactalbumin, 14.2 kDa.; soybean trypsin inhibitor, 21.0 kDa.; trpsinogen, 24.0 kDa., carbonic anhydrase, 29.0 kDa.; glyceraldehyde-3-phosphate dehydrogenase, 36.0 kDa.; egg albumin, 45.0 kDa.; bovine albumin, 66.0 kDa.)

Table 10. The distribution of proteins in blood from control locusts

Protein molecular weight	Percentage of total protein[a]			
(kDa.)	Day 1[b]	Day 2[b]	Day3[b]	Day4[b]
11.5	0.00	0.00	0.00	0.00
14.0	0.00	0.00	0.00	0.00
17.0	2.00	2.35	3.17	2.39
19.5	65.33	67.03	72.33	78.49
24.1	1.61	1.09	1.71	1.40
28.1	22.69	19.35	17.56	18.49
33.4	0.00	0.00	0.00	0.00
35.6	2.40	1.27	2.35	1.55
40.6	0.57	0.37	0.30	0.50
44.0	0.57	0.64	0.43	0.50
49.5	0.00	0.00	0.00	0.00
53.4	1.51	1.37	1.34	1.53
58.9	5.50	4.17	4.62	4.95
70.0	0.79	1.59	2.51	2.82

[a] Determined using a LKB laser densitometer.
[b] Figures reported as a percentage of the total protein per data point.

Table 11. The distribution of proteins in blood from locusts infected with *M. flavoviride* (330189).

Protein molecular weight (kDa.)	Percentage of total protein[a]			
	Day 1[b]	Day 2[b]	Day3[b]	Day4[b]
11.5	1.04 ± 0.14	1.82 ± 0.81	0.00	0.00
14.0	0.00	0.66 ± 0.10	0.00	0.00
17.0	3.81 ± 0.91	1.99 ± 0.62	3.65 ± 0.80	2.72 ± 0.65
19.5	71.55 ± 5.09	72.00 ± 3.19	78.04 ± 4.77	78.75 ± 0.99
24.1	0.51 ± 0.02	0.00	0.00	0.00
28.1	15.49 ± 4.44	11.03 ± 2.15	10.74 ± 1.25	4.36 ± 0.90
33.4	0.00	0.00	1.30 ± 0.20	1.46 ± 0.24
35.6	3.03 ± 1.10	2.20 ± 0.68	0.28 ± 0.08	1.09 ± 0.09
40.6	0.00	0.75 ± 0.18	0.00	0.00
44.0	0.52 ± 0.15	2.67 ± 0.58	0.54 ± 0.04	2.70 ± 0.72
49.5	0.65 ± 0.15	0.82 ± 0.29	0.00	1.02 ± 0.29
53.4	1.48 ± 0.20	0.28 ± 0.04	0.47 ± 0.01	0.00
58.9	2.61 ± 0.62	3.40 ± 0.78	3.15 ± 1.04	3.30 ± 0.39
70.0	1.76 ± 0.61	3.13 ± 0.59	3.63 ± 0.66	2.75 ± 0.14

[a] Determined using a LKB laser densitometer.
[b] Figures reported as a percentage of the total protein per data point. n=3; mean ± S.E. shown.

2.3.6. Haemocyte monolayers

Blood cell monolayers were prepared that could be employed to gain more insight into the cell populations that are involved in immuneresponses. These monolayers were used to stain for enzyme activity and to investigate binding charateristics.

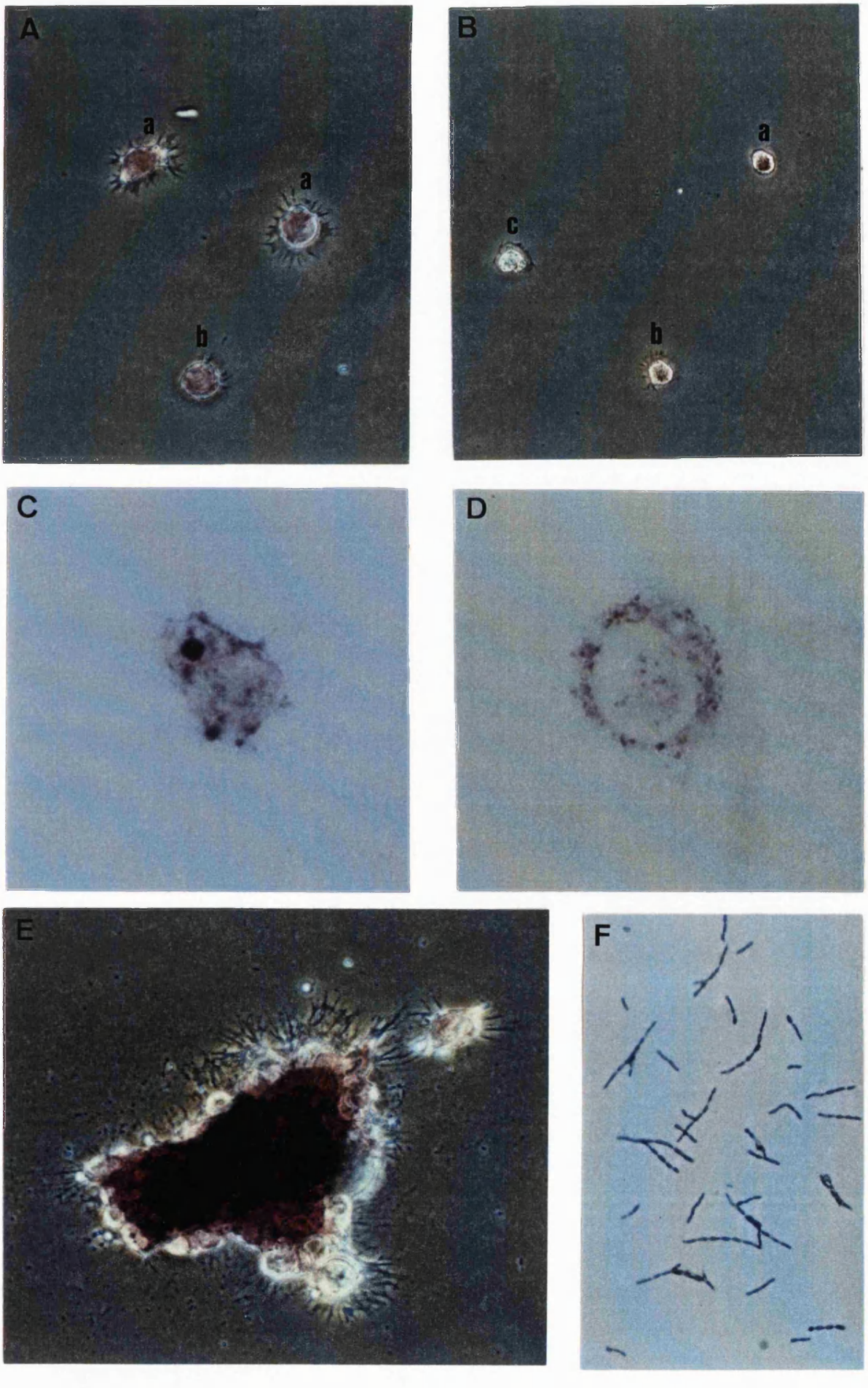
Figure 13a shows the results of staining cells for acid phosphatase activity. The cells stained either dark red or showed diffuse red staining (figure 13b). Table 12 shows the effect that infection had on the proportion of stained haemocytes. Whilst the proportion of stained GRs was maintained during mycosis, the number of acid phosphatase positive PLs increased significantly over the course of infection. Conversely, the number of COs decreases over the same period of time. The experiment could not be continued up to day 4 post inoculation as haemocytes from insects at this stage of infection failed to adhere in significant numbers to the glass slide.

If monolayers of naive haemolymph were prepared and overlaid with a 1 mg/ml solution of zymosan or laminarin (β,1-3-glucans) for 1 hour prior to staining for acid phosphatase activity, there was a change in the differential staining pattern (Table 13). The proportion of COs and GRs that were acid phosphatase positive decreased with both zymosan and laminarin. The PLs were unaffected.

If monolayers were incubated with blastospores or mycelial fragments, there was no change in the number of cells that stained for acid phosphatase activity (data not shown).

The increase in the proportion of PLs in infected blood staining for acid phosphatase activity may relate to nodule formation in which PLs are extensively involved. Figure 13e shows a nodule formed that is intensely red in colour indicating acid phosphatase activity. Of additional interest was

Figure 13 (overleaf): Photomicrographs of monolayers of haemocytes from *S. gregaria* and hyphal bodies of *M. flavoviride* stained for acid phosphatase (A) plasmatocytes (a) and granular cell (b) under 200x mag.: (B) coagulocytes showing intense (a), diffuse (b) and non-staining (c): (C) plasmatocyte showing large acid phosphatase rich vesicle after incubation with zymosan: (D) Diffuse staining coagulocyte undergoing "blebbing" with migration of aid phosphatase rich granules migrating to the periphery. (E) aggregation of haemocytes (nodule) on a monolayer stimulated with mycelial fragments. Note the large number of plasmatocytes on the periphery of the nodule: (F) hyphal bodies of *M. flavoviride* from the blood of a locust 4 days post inoculation.. Hyphae are staining positive for acid phophatase. (Magnification x400).


A
a
a
b
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C
D
E
F

Table 12. The effect of topical application of 7.5 x 10^4 conidiospores of *M. flavoviride* on haemocytes that stained positively for acid phosphatase activity in adult male *S. gregaria*[a].

Day after inoculation[b]	% Staining Cells[c]		
	CO	PL	GR
Control	87.60 ± 5.21	57.90 ± 3.00	100 ± 0.00
1	60.30 ± 4.70 [x]	39.70 ± 4.40 [x]	83.8 ± 11.8
2	49.50 ± 4.00 [x]	75.50± 10.80	100 ± 0.00
3	14.80± 5.82 [x]	83.94 ± 7.80 [x]	100 ± 0.00
4	N.A.[d]	N.A.[d]	N.A.[d]

[a] Figures reported plus or minus standard error. n = 12.
[b] Control insects were inoculated with cotton seed oil and maintained in conditions identical to infected insects and sampled at the same time points.
[c] Positive cells were intense staining cells; 200 cells were counted per monolayer.
[d] Not Attempted as there were less than 100 cells per monolayer.
[x] Significantly different from corresponding control value ($p<0.05$).

Table 13. The effect of β-1,3-glucans on acid phosphatase staining of blood cells.

Overlay	% intense staining cells[a,b]		
	CO	PL	GR
None	81.25 ± 1.10	61.75 ± 1.31	100.00 ± 0.00
Zymosan	33.25 ± 1.38[x]	61.75 ± 1.65	73.5 ± 0.75[x]
Laminarin	38.00 ± 1.47	55.00 ± 1.29[x]	64.75 ± 1.55[x]

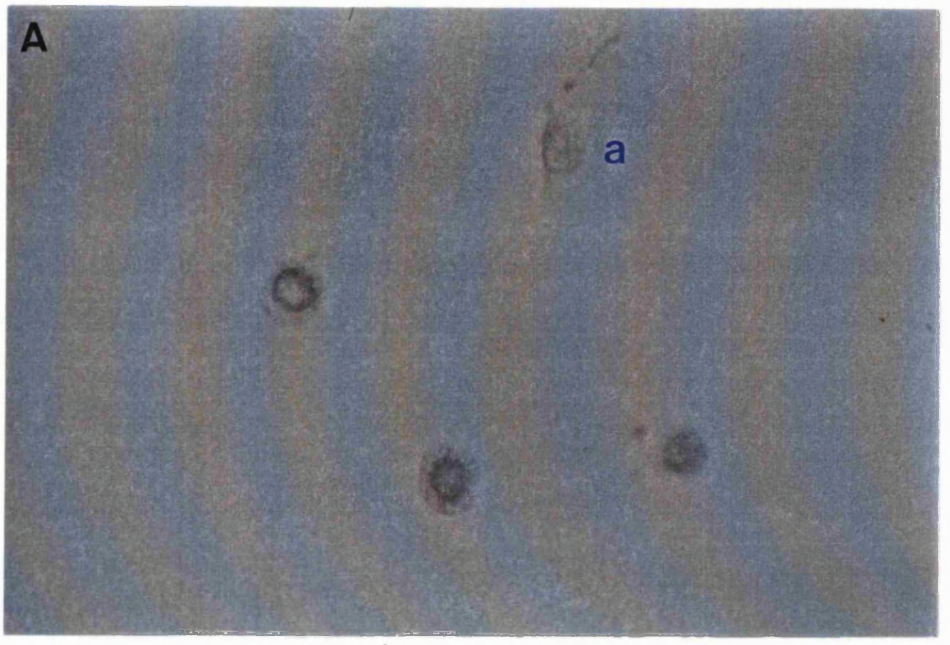
[a] Figures reported plus or minus standard error. n = 12.
[b] Positive cells were intense staining cells; 200 cells were counted per monolayer.
[x] Significantly different from corresponding control value ($p<0.05$).

the fact that the hyphal bodies observed in the blood on days 3 and 4 stained for acid phosphatase activity.

Monolayers also stained positive for Nitro Blue Tetrazolium (NBT) reduction indicating the presence of superoxide anions. These anions have been found in vertebrate blood cells in the process of phagocytosis. Staining for these ions in the cells of locust blood may suggest a role for reactive oxygen in the immune response. NBT reduction occurred in monolayers and was visualised as a purple/pale blue staining of the cytoplasm (figure 14). Staining was limited to the COs and GRs with 24.8± 2.9% of the CO population staining positive (table 14). If monolayers were overlaid with laminarin for 1 h, the cytoplasmic staining of the COs became more intense. Furthermore, small particulate formazan deposits were visible in some of these cells (data not shown). However, only 27.8 ± 5.6% of the CO population showed positive staining. If the monolayers were stimulated with a suspension of blastospores, the percentage of COs showing NBT reduction was 32.8 ± 3.8%. None of these values were significantly different from the control. Interestingly, it was observed that many of the COs and PLs lost the ability to adhere to the glass slides after the addition of blastospores. The staining profiles of individual haemocyte populations was not affected by infection as the percentage of cells staining did not alter significantly over the time course of mycoses (data not shown). In all monolayers SOD brought about a decrease in the intensity of the blue stain. However, complete inhibition of staining was not achieved. Attempts to quantify NBT reduction using the method of Anderson *et al.* (1992) were unsuccessful.

Monolayers were also used to investigate whether fungal particles would bind to haemocytes *in vitro*. Figure 15 shows that if protoplasts were overlaid on a monolayer, the cells would not bind to them. On the

Figure 14 (overleaf): Photomicrographs of a monolayer of coagulocytes from an infected *S. gregaria* 2 days post-inoculation exhibiting NBT reduction. (A) bright field optic demonstrating positive staining cells and a non-staining cell (a); (B) Phase contrast of the same monolayer identifying the cell types. (Magnification x400).


A
a

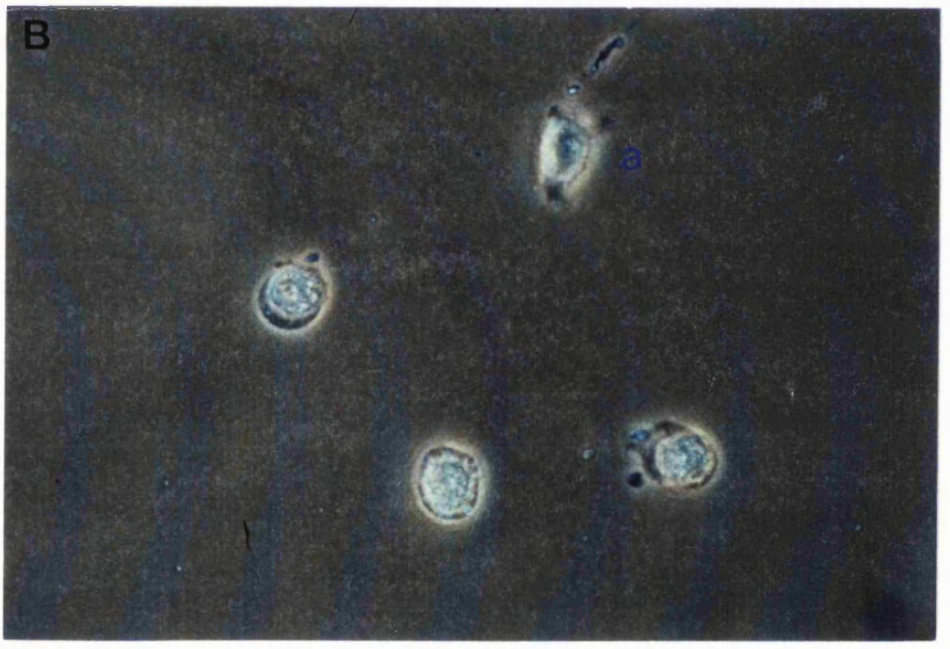

B
a

Table 14. The effect of β-1,3-glucans and blastospores on NBT reduction in locust blood cells.

Overlay	% staining for O_2^- [a,b]		
	CO	PL	GR
None	24.80 ± 2.90	0.00	15.60 ± 1.06
Laminarin	27.80 ± 5.60	0.000	17.20± 0.86
Blastospores	32.80 ± 3.80	0.00	16.10 ± 0.98

[a] Figures reported plus or minus standard error. n = 12.
[b] Positive cells were intense staining cells; 200 cells were counted per monolayer

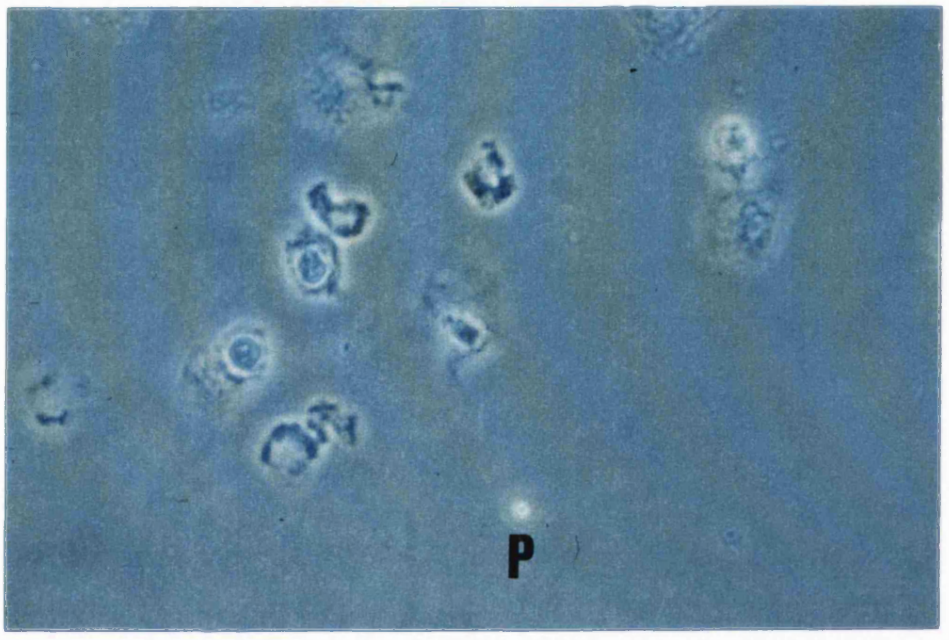


Figure 15. Photomicrograph of a haemocyte monolayer overlaid with 10^8 protoplasts/ml in phosphate buffered saline. Note the lack of adherence of protoplasts (P) to blood cells. (Magnification x400)

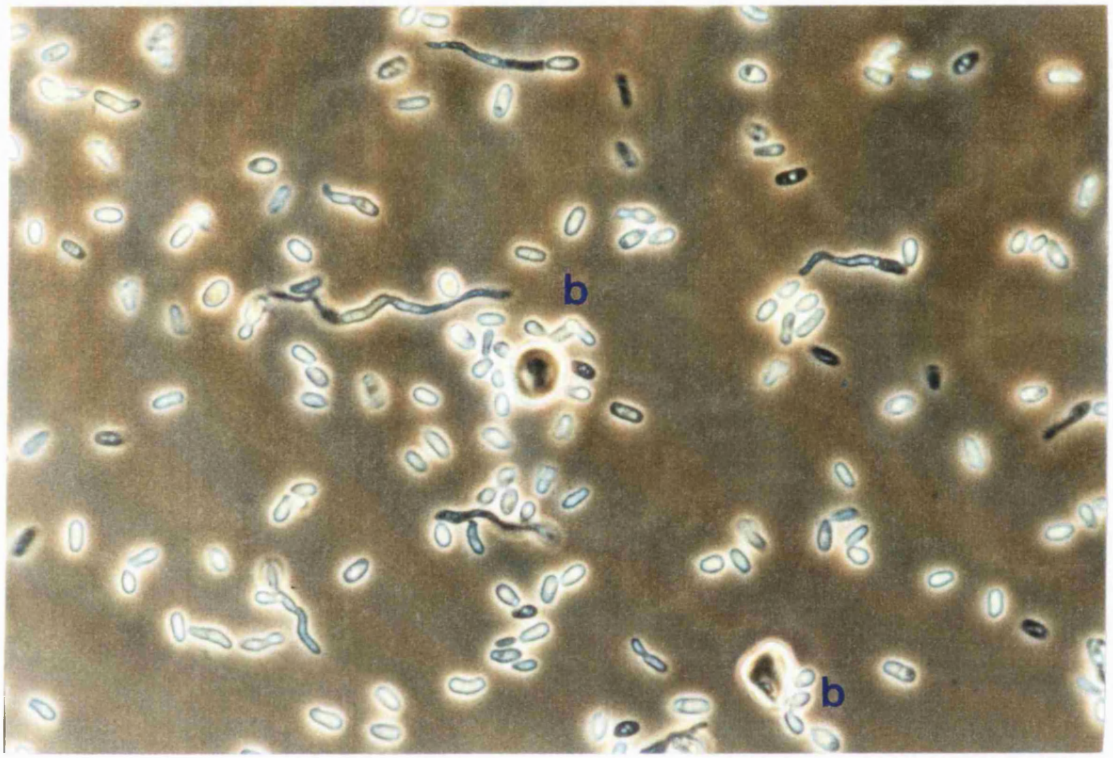


Figure 16. Photomicrograph of a haemocyte monolayer overlaid with 10^8 blastospores/ml in phosphate buffered saline. Note the adherence of blastospores (b) to blood cells. (Magnification x400).

Table 15. The percentage of blood cell types that bind fungal bodies.

Overlaid Particle	Percent of haemocytes Binding[a]			Percent haemocytes non-binding
	Coagulocyte	Plasmatocyte	Granular cell	
Blastospore	13.25 ± 1.26	25.80 ± 0.86	0.00	60.95
Mycelial fragments	9.15 ± 1.02	33.16 ± 2.56	0.00	57.69
Protoplast	0.00	0.00	0.00	100

[a] Figures reported plus or minus standard error. n = 12; 200 cells were counted per monolayer.

other hand, overlaid blastospores and mycelial fragments bound to haemocytes (Figure 16).

To quantify the number of each cell type bound to fungal bodies, 200 cells were counted and their binding characteristics observed (Table 15). Both blastospores and mycelial fragments were observed to bind to the haemocytes. Proportionately, the PLs bound to the fungal material in significantly larger numbers than to the COs.

2.4. Discussion

The defence responses of insects to microbial pathogens has been researched extensively (for recent reviews see Ratcliffe, 1993; Hultmark, 1993). However, it has become evident from the literature that most of the immunological events observed have been after the injection of microbial material rather than following naturally induced infections. This study has dealt with the effects that the topical application of a virulent isolate of *Metarhizium* spp. has on the immune responses of the desert locust, *S. gregaria.* This is the first extensive study of the immunological events elicited by the topical application of any *Metarhizium* species. In this study it was shown that *M. flavoviride* could colonise the host blood within 2/3 days of inoculation and kill the insect by 4 days. The time of death corroborates with data reported elsewhere and supports the *in vitro* evidence that the triple sulfa drugs supplemented to the locust diet had no effect on disease development. Hyphal bodies were only found in the blood 3 days after inoculation. However, when a large conidial inoculum was used, protoplast-like structures were seen in the blood of infected individuals only 2 days after application. It is well established that Entomophthoralean fungi can grow as protoplasts in the blood of their hosts (Dunphy and Nolan, 1989). This growth form may enable the fungus to evade the host immune system. However, it is only recently that a Deuteromycete (*B. bassiana)* has been shown to produce protoplast-like bodies *in vivo* (Pendland *et al.*, 1993). In the present case, *M. flavoviride* appears to switch from a protoplast form of growth to a walled-hyphal body form between 2-3 days after inoculation. This is an important observation and needs confirmation.

In this study, the total haemocyte count (THC) during infection declined following an initial increase. Declines in THC in insects during

microbial infection have been observed many times but most work has been done in response to bacterial infections (see Ratcliffe *et al.*, 1985). Hung and Boucias (1992) observed a decline in the THC of larvae of *Spodoptera exigua* in response to injection of conidiospores of *B. bassiana.* In Acridids, Bidochka and Khachatourians (1987a) reported that there was a decline in the THC of *M. sanguinipes* in response to the injection of *B. bassiana* spores. In each of the above cases, an initial increase in the THC was not observed. This phenomenon may not occur where infections were brought about by injection, thus by-passing the natural route of entry. The increase in THC observed here could be due to the mobilisation of cells from the haemocoel lining and may be caused by factors released from the integument during fungal penetration. One candidate molecule could be the so-called wound response factor suggested by Gunnarsson (1988b). However, it is not clear why Gunnarsson (1988a), who also observed a reduction in the THC in desert locusts in response to the topical application of spores, failed to see an initial increase in THC. However, he used *M. anisopliae* rather than *M. flavoviride.* The subsequent decline in THC observed in this study may result from the formation of nodules, the formation of granulomas on the basement membrane of the haemocoel lining or the result of cytotoxic events mediated by soluble factors produced by the fungus. Indeed, Mazet *et al,* (1994) have found that *B. bassiana* produces a toxic metabolite *in vivo* during infection of *S. exigua* larvae. This, unidentified protein, reduced the activity of haemocytes *in vitro.*

The identification of haemocytes here concurs with that of Lackie *et al.,* 1985). However, their differential haemocyte count (DHC) indicated there to be more PLs than COs. Lackie *et al.* (1985) performed their DHC on locusts that had been anaesthetised using carbon dioxide and observed that there were differences between insects anaesthetised this

way and those that were chilled on ice. In conjunction with the results presented here, previous observations from this laboratory indicate that the proportion of cells present in the blood of locusts are in the order CO>PL>GR (Dillon, Unpub.). Studies on the individual haemocyte types during infection show an increase in the proportion of COs and decrease in the proportion of GRs and PLs. One interpretation of this is that the PL sub-population became involved in the immune response and gradually disappeared from circulation during mycosis. This concurs with the observation made by Gunnarsson (1988a) that this cell type declines during pathogenesis. Chain and Anderson (1983b) have described a humoral "plasmatocyte depletion factor" in the blood of *G. mellonella* which signals the PLs to migrate to regions of the body that require nodule and capsule formation. This factor has not been described in Acridids but there is a possibility that it occurs because the proportion of PLs declines during infection. The slight decline in GRs observed may be due to their involvement in the latter stages of nodule formation. Classically, nodule formation has been reported to involve the GRs (see Gotz and Boman, 1985). However, the decline in the proportion of GRs in the latter stages of infection here suggests that they are not involved in nodule formation. Furthermore, the number of nodules formed is small and this may not exert a notable effect on the number of blood cells recruited. Alternatively, the cells may be depleted by lysis due to toxic metabolites produced by the fungus. This notion needs further clarification. The increase in the proportion of COs remains unexplained except that they maybe the last cells to be mobilised as they may lack an immune function. The cells that produce pPO have not been identified and it is possible that the granule-containing COs are responsible for the proenzyme. If this is the case, if the proportion of COs increase as result of the PLs being depleted, one would expect no change in the total pPO activity. This was not observed as the

pPO activity increased during infection implying synthesis of the proenzyme.

In this study, an increase in the number of haemolymph nodules was observed in *S. gregaria* after infection with the maximal number observed only 1 day after inoculation. Of interest was the observation that nodules were present in control insects. Low levels of nodules may be present in response to wounding of the insect. Another explanation for their occurrence would be that they are stress-induced. Stress can be referred to as a "state manifested by a syndrome, or bodily changes, caused by some force, condition or circumstance in or on an insect or on one of its physiological or anatomical state" (Brey, 1994). Thus, stress in insects can be caused by a multitude of biotic and abiotic factors such as crowding, handling, pathogenic infection, extreme temperatures, metamorphosis and mechanical injury. Infection of *S, gregaria* with *M. flavoviride* and the handling and housing of insects during experiments can represent stressful conditions for the host. The early response to infection manifested in the form of increased number of nodules could be a direct response to a signal, possibly a factor(s) released from the cuticle in response to penetration. The subsequent decline in nodule numbers may be due to them reaching a certain critical size and dropping out of circulation and lining the body cavity (Rowley *et al.*, 1987). Brookman *et al.* (1989a) observed large numbers of nodules lining the body cavity of *L. migratoria* in response to bacterial injection. In the case of *S. gregaria*, no nodules were observed lining the haemocoel in response to natural fungal infection. This may be simply because fewer nodules are formed.

The nodules observed in this study showed variable size. However, earlier during infection, the size of the nodules was, in general, smaller than those observed later on in mycoses. It is possible that soluble elicitors of nodule formation are released during penetration. This would result in

small nodules being formed (Gunnarsson and Lackie, 1985). As macromolecular elicitors such as the fungus access the haemocoel, larger nodules are promoted. Although nodules may be induced by the presence of the fungus, there were no indications that nodules were forming around fungal hyphal bodies. Many of the nodules were brown in colour indicating melanisation had occurred. This is a common phenomenon in encapsulation as phenoloxidase (PO) is active in forming a melanotic sheath around the invading organism (see Gotz and Boman, 1985).

The level of PO in control insects was seen to increase significantly and then decline over the course of the experiment. This increase in enzyme activity is possibly related to stress. Indeed, the number of nodules increased early on in control insects which links well with increase in PO production. Phenoloxidase levels decreased over the course of infection in experimental insects without an intial rise. This would be logical as phenoloxidase will be used up melanising foreign bodies. Gillespie and Khachatourians (1992) observed an initial increase in PO levels in *M. sanguinipes* in response to the injection of spores of *B. bassiana*. This was followed by a subsequent decline in enzyme activity followed by a secondary rise. If insects were wounded by piercing the cuticle, a primary increase in PO levels was seen but not a secondary one. Phenoloxidase indiscriminately generates quinones which are highly reactive and cross-link with tyrosine residues on any protein and, unfortunately, will become cross-linked itself and be retained in melanotic capsules. An increase in the amount of prophenoloxidase (pPO) was observed in this study which is interesting. The decline in PO levels and concommitant increase in pPO levels indicates that the proenzyme is being produced but not being activated. One possible explanation for this is that the ability of the host immune system to recognise non-self has been altered.

The fungus could possibly produce an inhibitor to prevent this activation. Huxham *et al.* (1989) found that PO activity was suppressed by the fungal cyclic depsipeptide toxin, destruxin. Destruxins are produced by *M anisopliae.* but not by *M. flavoviride* (Kershaw, 1993). Thus, if the fungus is inhibiting activation of pPO, then it must be by some other mechanism. A protease inhibitor would prevent activation since pPO is activated by a serine protease (Sugumaran and Kanost, 1993). This scenario seems unlikely as the fungus itself produces a battery of serine proteases including a chymotrypsin-like protease (see Samuels and Patterson, 1995). Alternatively, the fungus could prevent binding of the β-1,3-glucan of the fungal cell wall to its receptor protein. The activation of pPO is dependent upon a recognition system, for a fungus this probably involves a β-1,3-glucan binding protein (Duvic and Soderhall, 1990). This in turn activates a pPO-activating enzyme which cleaves pPO to the active PO form. If this pathway is, in some way, inhibited, PO will not be activated.

In this study, lysozyme activity was monitored during infection and observed not to alter appreciably. Similarly, in *S. exigua* larvae injected with *B. bassiana*, no observable alteration in the haemolymph lysozyme titers occurred (Boucias *et al.*, 1994). Furthermore, infection with *B. bassiana* did not limit the insects ability to mount a response to a secondary injection of LPS. This may have some significance as lysozyme is an antibacterial enzyme that cleaves N-acetylmuraminic acid which is found in bacterial cell walls but not in fungi. This suggests differential activation of the immune response rather than an all or nothing phenomenon. It seems reasonable that lysozyme need not be induced against a microorganism that it can have no action against.

The acid phosphatase levels in insects initially increased after infection and subsequently declined to levels significantly higher than

control animals. *M. flavoviride* produces acid phosphatase *in vitro* (data not shown) and fungal enzyme may make a contribution to acid phosphatase levels later on in infection. However, *M. sanguinipes* artificially infected by injection of *B. bassiana* showed slight changes from controls but this was not significant as the fluctuations occurred in the haemocytes as well (Vincent *et al.*, 1993).

This study shows that the protein components of the haemolymph change during infection even though the protein concentration as a whole does not vary appreciably. Studies on protein changes in the haemolymph of diseased insects has been reviewed recently (Beckage, 1993). However, much of the work involved parasitic wasps or worms (Beckage *et* al., 1989; Beckage and Kanost, 1993, Harwood and Beckage, 1994; Wattan *et al.,* 1992). Indeed, in *M. sexta*, new host proteins are synthesised in response to parasitism, heat or cold shock, pathogenic infection or physical trauma and injury (Beckage, 1993). In *S. gregaria*, there are changes in the activity of acid phosphatase and PO in blood plasma during infection. However, the molecular weights of these enzymes are not known and thus it is not possible to link them with any of the protein bands on the electrophoretic gels. The low molecular weight (11.5 kDa) protein which makes a transitory appearance in infected blood (days 1 and 2) could be a host signalling molecule or cytokine responsible for orchestrating early events in the immune response. Alternatively, it may be the wound response factor identified by Gunnarsson (1988b). Its early appearance makes it unlikely to be a fungal protein. Beckage and Kanost (1993) found parasitism-induced reductions in specific proteins such as arylphorin and the serpins in *M. sexta* parasitised with *Cotesia congregata.* In the present work, at least 2 protein bands (24.1 kDaand 28.1 kDa) declined in quantity during infection. The identities of these are not known but from their molecular weights they are unlikely to be either components of arylphorin

or a serpin. A decline in titre of proteins in the blood may reflect changing patterns of protein synthesis in mycosed insects, in part due to reduced feeding. Alternatively, proteins may be hydrolysed by pathogen proteases, though no evidence exists for the presence of the fungal chymoelastase PR1 in the blood of infected locusts (see chapter 4). Indeed the only protein with anywhere near the molecular weight of PR1 (28.6 kDa.) is the 28.1 kDa. protein which was present in control blood and declined in infected insects during mycosis. Two proteins appear either later in the infection process (33.4 kDa.) or throughout the infection (49.5 kDa.) but not in controls. These could be either fungus or host derived. The appearance of the 33.4 kDa. protein after 3 days is of particular interest. This time coincides with the appearance of significant quantities of fungus in the blood of locusts. It is the same molecular weight as the α-glucosidase of *M. anisopliae* which has been found in the blood of *M. anisopliae*-infected *M. sexta* 3 days after inoculation (Cobb, Pers. Comm.). If the 33.4 kDa. protein is a fungal α-glucosidase, this could account for the decline in carbohydrate concentration in the plasma of mycosed locusts 3 days after inoculation (Seyoum, 1994).

Monolayers of cells from *S. gregaria* were established and stained for acid phosphatase showing that the enzyme was present in haemocytes, as well as the plasma. A similar observation has been recorded in other insects (Chain and Anderson, 1983a; Miranpuri *et al.*, 1991). The enzyme is an important lysosomal marker protein in mammalian phagocytes and fluctuations in its activity gives a good indication of which cell types are involved in phagocytosis. There was an initial decline in the proportion of PLs in infected blood that were acid phosphatase positive followed by an increase 2-3 days after inoculation. This suggests that more cells become phagocytically competent. This is consistent with the fact that β-1,3-glucans caused an increase in the number of phagocytically competent

haemocytes (Gunnarsson, 1988b). Although overlays of β-1,3-glucans significantly reduced the proportion of COs and GRs on monolayers staining for acid phosphatase, there was no change in acid phosphatase staining in PLs. This may indicate that changes in acid phosphatase activity and phagocytic competence in PLs are brought about by cytokines released from other cells rather than by direct response to fungal antigens.

This study showed that the COs and GRs were the only cells to stain for NBT reduction. This is important because the PLs are the major phagocytic cell in the haemolymph. In vertebrates, superoxide anions, which are toxic to microbes and which reduce NBT, are produced by phagocytic cells. The lack of NBT staining by PLs suggests that the NBT reduction observed in other cells may be due to other reducing agents produced in the haemolymph. This possibility was supported by the failure to completely inhibit NBT reduction with superoxide dismutase. Infection had no significant effect on the proportion of COs and GRs that reduced NBT.

In this study, the binding of haemocyte sub-populations to fungal fragments was investigated. Protoplasts did not bind to blood cells. This is not unexpected as protoplasts are devoid of β-1,3-glucans on their cell surface and would not activate the immune system. Protoplast-like bodies are produced by *B. bassiana in vivo* in *S. exigua* larvae (Pendland *et al.*, 1993) where they were shown to avoid the immune system and circulate freely. The occurrence of protoplast-like bodies in the blood of locusts infected with *M. flavoviride* has been shown here. Growth of the fungus initially as protoplasts may prevent the attentions of the haemocytes but early changes in the plasma proteins, enzyme levels and the proportion of blood cell types suggests the immune system has been activated. This response may be due to the effects of injury (passage of fungus through

the cuticle) or the host may detect the fungus in its pre-penetration existence on the surface of the cuticle.

In vitro produced blastospores and hyphal bodies can bind to blood cells in monolayers. However, *in vivo* the fungal bodies in the blood of *S. gregaria* were never found associated with haemocytes. This may suggest a difference in wall composition between *in vitro* and *in vivo* produced hyphal bodies/blastospores. Alternatively, the fungus may produce a toxin(s) that inhibits binding of haemocytes to fungal cells. In *S. exigua* Hung *et al.* (1993) showed that haemocytes taken during infection are immunocompetant and able to ingest particles.

This study has investigated several components of the immune system of *S. gregaria.* Of particular importance is the fact that immune responses were elicited using the topical application of spores of *M. flavoviride.* In conclusion, it is apparent that there are two stages to infection with respect to the events occurring in the blood of infected insects. During the first stage, in the first 2 days after inoculation, there was an increase in THC, the number of nodules and levels of enzyme activity. All of these parameters were shown to increase above control levels when there was no or only a very minor presence of fungus in the blood. This suggests that there may be soluble factors released by the host that activate the immune system. Alternatively, fungal metabolites may leak into the blood and activate the immune system. An all-or-nothing response appears to be mounted with a uniform increase in all parameters investigated. The second stage observed in this study was when the fungus had entered the haemocoel and replicated extensively (3-4 days after inoculation). At this time, the parameters of the blood borne defence system investigated, were at levels significantly below those earlier in mycosis. This may be because the immune system has now been overcome by the fungus or fungally-derived metabolites

Chapter 3

3.0. Purification and partial characterisation of protease inhibitors from the haemolymph of *Manduca sexta* and *Schistocerca gregaria.*

3.1. Introduction

Protease inhibitors form a significant proportion of the total proteins in vertebrate sera (Laskowski and Kato, 1980; Travis and Salvesen, 1983). Extensive studies in mammalian systems have identifiedthe primary rol

activity of a wide diversity of metabolic processes such as blood clotting, complement activation and proteases released from neutrophils (Heimberger, 1975; Travis and Salvesen, 1983).

In insects, protease inhibitors may also be essential in the regulation of protein metabolism (Eguchi, 1993) and have been found in diverse insect species (see Polanowski *et al.*, 1992). Primarily inhibitory activity toward chymotrypsin and/or trypsin has been focused on and a number of inhibitors have been purified and characterised from *Bombyx mori* (Eguchi *et al.*, 1982), *Anticarsia gemmatalis* (Boucias and Pendland, 1987) and *Manduca sexta* (Kanost, 1990a; Ramesh *et al.*, 1989). Much of the research has been concerned with holometabolous insects (i.e. those that show complete metamorphosis). However, evidence suggests that protease inhibitors are present in insects from a whole range of species including Coleoptera, Lepidoptera, Hymenoptera, Orthoptera, Dictyoptera and Hemiptera (Polanowski *et al.*, 1992).

3.2. Insect Protease Inhibitors.

Inhibitors were first identified in whole body or tissue homogenates of insects such as *Drosophila melanogaster*, *Leucophaea maderae* and *Aedes aegypti* (see Eguchi, 1993). However, Suzuki and Natori (1985, 1986) found several inhibitors toward a haemocyte protease in the haemolymph of *Sarcophaga peregrina*, one of which increased in concentration after ecdysis to 3rd instar and during puparium formation. Multiple inhibitors have been found also in the blood of *B. mori* and *M.sexta*. In the latter insect, Ramesh *et al.* (1988) isolated two low molecular weight Kunitz-type inhibitors from 5th instar larvae. Both were pH and heat stable and, after N-terminal amino acid analysis, were shown to have homology with bovine pancreatic trypsin inhibitor. Kanost *et al.* (1989) isolated a cDNA clone from a fat body cDNA library in *M. sexta*. It had an open reading fame coding for a 392 residue protein and a deduced molecular weight of 43.5 kDa. Subsequently, the authors isolated a 47 kDa. glycoprotein from the blood of *M. sexta* larvae which had an amino terminal sequence identical to that deduced from the cDNA clone. This protein strongly inhibited porcine pancreatic elastase and bovine chymotrypsin. Three more serine protease inhibitors were subsequently isolated from larval blood (Kanost, 1990a). Two of the four were specific for chymotrypsin, one for trypsin and one for elastase. Each inhibitor had a similar isoelectric point, molecular weight, amino acid composition and amino terminus sequence. The author suggested that these inhibitors were members of a gene family and related to the serpin (serine protease inhibitor) gene super family found in mammalian systems (Carrell and Travis, 1985). Takagi *et al.* (1990) have sequenced an antitrypsin cDNA from *B. mori* and found it to be 56 % homologous with one of the serpins found in *M. sexta*. However, the sequence around the reactive site and

inhibition profiles were notably different. This phenomenon is plausible as rat liver serpins show high degrees of homology overall but not around their reactive sites (Hill and Hastie, 1987). Furthermore, Kanost (1990b) observed the presence of inhibitors of elastase, chymotrypsin and trypsin in the blood of *Drosophila melanogaster*. Based upon experiments showing weak cross-reactivity with anti-serpin antibodies, it was suggested that these proteins are serpins as well.

During the purification of the four major serpins from *M. sexta*, Kanost (1990b) observed several other, less abundant, proteinase inhibitors of the same size. Immunoblot analysis revealed that there were approximately ten acidic serpins present in the blood indicating multiple, antigenically related, serpins. Analysis at the molecular level by Jiang *et al.* (1994) showed the presence of 38 cDNA clones for serpins. They were identical in sequence except for a region encoding the carboxy terminal 40-45 residues which includes the reactive centre region. This region is encoded by different versions of the 9th exon which was found to be present in 12 variable forms. It was suggested that the insect serpin gene has resulted from duplication and sequence divergence of only the exon encoding the reactive site. This alternative pre-mRNA splicing results in inhibitor diversity and the potential to regulate a variety of proteinases.

The proteinases that these serpins inhibit *in vivo* are, as yet, not known. Kanost (1990a) implicated them in the regulation of prophenoloxidase activation, protection for insects from proteases secreted by pathogens and accidental leakage of gut or moulting fluid proteases. Consistent with this hypothesis, haemolymph phenoloxidase activity of *M. sexta* larvae injected with anti-serpin antibodies, was significantly higher than that for insects injected with saline (Kanost, 1990b). Further evidence comes from the observation that the serpins inhibit midgut trypsin and chymotrypsin *in vitro* (see Kanost, 1990a). Furthermore, Kanost (1990a)

has also suggested that some protease inhibitors may have lost the function to inhibit target enzymes but act with a different function This is the case with angiotensin, corticosteroid-binding globulin and thyroxin binding globulin.

Eguchi and co-workers have performed extensive studies on the protease inhibitors present in *B. mori*. *B. mori* haemolymph inhibited bovine chymotrypsin and trypsin as well as proteases from the haemolymph, midgut tissue and alimentary canal of the silkworm (Eguchi *et al.*, 1982). Eguchi *et al.* (1984) established that these protease inhibitors consisted of a family of isoinhibitors controlled by co-dominant alleles. The highest inhibitory activity was seen in the mature larvae (spinning period) and one function of the protease inhibitors may be to inactivate the proteases discharged by apoptosis during metamorphosis (Eguchi *et al.*, 1986). Extensive tissue degradation occurs in *B. mori* larvae in the pharate pupal and early pupal stage (Eguchi and Kanbe, 1982) and there is a good correlation between haemolymph inhibitor activity and this metamorphic change. Evidence is consitent with the regulation of *B. mori* inhibitors by hormones that control growth and moulting (Matsui and Eguchi, 1991).

One of the key roles suggested for protease inhibitors is the protection of insects from the proteases secreted by pathogens, particularly fungi, which invade through the integument. Inhibitors from the blood of *B. mori* are effective against proteases from the fungal pathogens *Aspergillus melleus* and *Beauveria bassiana* (see Eguchi, 1993). Interestingly, inhibitory activity against fungal proteases was found in the integument of *B. mori*, the first barrier to infection. One fungal protease inhibitor, FPI-F, showing high activity in both the haemolymph and integument, suppressed germination of conidia and germ tube development of *B. bassiana* (Yoshida *et al.*, 1990). Thus, it may serve as an important mediator in the defensive functions of *B. mori*. The inhibitor was purified

and found to inhibit subtilisin and fungal proteases strongly and, only to a lesser extent, chymotrypsin and trypsin (Eguchi *et al.*, 1993a). Interestingly, the inhibitor showed no significant homology to any other known protease inhibitors (Eguchi *et al.*, 1994).

Inhibitors of fungal proteases have been reported in the blood of insects other than the silkworm (Kucera, 1982, 1984; Boucias and Pendland, 1987). Boucias and Pendland challenged *A. gemmatalis* with intrahaemocoelic injection of the entomopathogen *Nomuraea rileyi* and found that levels of inhibitory activity against trypsin increased 48 h after treatment. Furthermore, they observed that the inhibitor prevented both conidial germination and germ tube development of *N. rileyi.*

Hall and Soderhall (1983) described a protease inhibitor in the crayfish, *Astacus astacus* and suggested that the inhibitor present in the cuticle originated from the haemocytes. Interestingly, this inhibitor showed activity towards the partially purified protease from the crayfish pathogen, *Aphanomyces astaci*, but not the proteases from two saprophytic members of *Aphanomyces* spp.

A common theme is that the strong chymotrypsin inhibitory activity in the haemolymph may protect tissues from endogenous digestive proteases which may leak into the haemolymph from the alimentary canal (Eguchi *et al.*, 1982). This would be the case in insects infected by bacteria or viruses and during apoptosis that accompanies metamorphosis. The changes in titre of the fungal protease inhibitors at metamorphosis were much less than those for chymotrypsin inhibitors (Yamashita and Eguchi, 1987) and the authors suggested that, unlike the latter, the former have no role in development.

The presence of large molecular weight inhibitors has been shown in crustaceans such as crayfish and lobsters (for review see Armstrong and Quigley, 1992). These inhibitors are termed α_2-macroglobulins (α_2-M) and

were originally isolated from the plasma and body fluids of vertebrates (see Laskowski and Kato, 1980). The mode of inhibition of these glycoproteins is unique and quite unlike that of the active site inhibitors described above. As the α_2-M encounters the target enzyme, it physically "ensnares" it resulting in a conformational change in the inhibitor's shape. This change results from the protease cleaving a single peptide bond in the so-called "bait" region (a specific segment of the inhibitor that is susceptible to proteolytic cleavage) of the α_2-M. The active site of the entrapped enzyme is free to react with substrates small enough to enter the 'molecular cage'. However, large substrates, such as proteins are excluded from the active site of the enzyme. This ability of α_2-M-protease complexes to hydrolyse small substrates but not large ones provides a basis for identifying this type of inhibitor (Barrett and Starkey, 1973).

The function of these inhibitors is thought to be to remove potentially destructive proteases from the body fluids. What is not understood is why there appear to be both active-site inhibitors and α_2-M inhibitors present in the blood. However, one clue may be the fact that in the horseshoe crab, the active site protease inhibitors are present in the blood cells whilst the α_2-M are in the plasma. α_2-M type inhibitors have not been identified in insects thus far.

The prophenoloxidase (proPO) activating system is considered to be a key component responsible for defensive functions in arthropods (see chapter 1; Ratcliffe *et al.*, 1984; Soderhall and Smith, 1986b). Sugumaran *et al.* (1987) found that serine protease inhibitors in the blood of *M. sexta* and *Sarcophaga bullata* prevented the activation of proPO by inhibiting the activating protease. Jiang *et al.* (1994) have also suggested that the serpins would be good candidates for proPO regulation as the system requires regulation in a similar way to the blood clotting system of vertebrates. Two proPO inhibitors have been isolated from *Locusta*

migratoria (Boigregrain *et al.*, 1992). They were very small (35 and 36 amino acid residues long respectively) and showed very strong inhibitory activity to α-chymotrypsin and pancreatic elastase. Sequence analysis showed that these inhibitors were identical to two proteins isolated from the brain of *L. migratoria* (Nakakura *et al.*, 1992). Subsequently, Kromer *et al.* (1994) have cloned the cDNAs of these inhibitors and, via Northern blot analysis shown that the gene encoding these precursor was mainly transcribed in the cells of the fat body. The role that these proteins play *in vivo* remains unexplained although evidence is consistent with several classes of protease inhibitor in insect haemolymph and it is still possible that individual inhibitors have a number of functions.

Information on protease inhibitors in Acridids is sparse. The aim of this study was to purify and characterise protease inhibitory activity against the chymoelastase PR1 enzyme in the haemolymph of *S. gregaria.* If present, the inhibitory activity was to be partially characterised. It has been suggested that a role for the protease inhibitors in lepidopteran insects such as *B. mori* and *M. sexta* is the inhibition of proteases produced by microorganisms, especially entomopathogenic fungi. Therefore, an additional goal of the present work was the identification of an inhibitor in *M. sexta* with activity against the *Metarhizium* protease, PR1.

3.2 Materials and Methods.

3.2.1. Chemicals.

All chemicals were analytical grade and purchased, unless otherwise stated from Sigma or BDH. HPLC grade solvents were obtained from Rathburns.

3.2.2. Insects

3.2.2.1. Maintenance.

Adult males of *S. gregaria*, at least 14 days after final ecdysis were used. Locusts were maintained as described in chapter 2.

Fifth instar larvae of *Manduca sexta* (Lepidoptera: Sphingidae) were used for the collection of blood. These insects were maintained at 25^{o}C, under a 17h light, 7h dark photoperiod on a agar-based artificial diet using standard procedures (see appendix; Bell and Joachim, 1976).

3.2.2. Inoculation of desert locusts with *Metarhizium flavoviride.*

Adult *S. gregaria*, 14 days after final ecdysis, were infected with *M. flavoviride* and maintained as described previously (see section 2.2.5)

3.2.3. Partial purification of proteins from the haemolymph of *S. gregaria and M. sexta* with inhibitory activity against PR1

3.2.3.1. Collection of blood and initial purification of protease inhibitor.

Blood was collected from locusts as described in section 2.2.6.1. The cells were removed by centrifugation at 13000 rpm for 4 min at 4°C. An aliquot (50 µl) was removed and added to an equal volume of anticoagulant buffer. This fraction (plasma) was stored at -20°C until used. The haemocyte pellet was resuspended in distilled water, vortexed vigourously for several minutes before being centrifuged at 13000 rpm for 4 min at 4°C. The supernatant was checked for particulate matter and, if none was present stored at -20°C. This fraction was called the haemocyte lysate. An inhibitor of PR1 activity was found in the plasma fraction of the haemolymph.

For the purification, blood was collected from locusts irrespective of age. Three ml of haemolymph was pooled into an equal volume of anticoagulant buffer over ice and subsequently centrifuged at 4°C and 13000 rpm for 10 min. The supernatant was collected and diluted 4 times with ice cold anticoagulant buffer. The diluted plasma was partitioned with 1 volume of chloroform for 10 min and centrifuged at 8000 rpm and 4°C for 15 min. The upper, aqueous layer resulting from centrifugation was decanted and the volume measured. The plasma extract was then mixed with 50% (v/v) saturated ammonium sulphate solution and left to stand on ice for 60 min. The sample was then centrifuged at 8000rpm and 4°C for 15 min. The resultant supernatant contained the inhibitor and was filtered through a 10 kDa cut-off membrane (Amicon) under pressure until a final volume of approximately 2 ml remained. This was then resuspended in 20

ml 10 mM Tris-HCl (pH 7.0) and re-filtered as before. This was repeated twice until the residue was resuspended in a 5 ml Tris-HCl and frozen at -20°C until used.

An inhibitor of PR1 was also shown in the blood of Vth instar larvae of *M. sexta*. Blood was collected from 18 larvae in 5 ml ice cold bleeding buffer (20mM Tris-HCl; 0.01% phenylthiourea; 0.01% sodium azide; Ramesh *et al.*, 1988). The blood was then centrifuged at 13500 rpm and 4°C for 5 min in a Sorvall RC5B centrifuge. The volume of supernatant was measured and mixed with an equal volume of carbon tetrachloride to remove lipid. After vigorous shaking, the mixture was left to stand on ice for 10 min before being centrifuged at 8000 rpm and 4°C for 10 min. The upper aqueous layer was then removed and mixed with saturated ammonium sulphate so that the final concentration of salt was 30% (v/v). A few crystals of phenylthiourea were added and the mixture was allowed to stand on ice for 60 min before centrifugation at 8000 rpm and 4°C for 10 min. The supernatant was collected and dialysed against a 200 volumes of 10 mM Tris-HCl (pH 7.0) at 4°C overnight. The dialysate was then concentrated under high pressure using a YM5 membrane filter (Amicon) until a final volume of approximately 10 ml was obtained.

3.2.3.2. Anion exchange chromatography.

Ion exchange chromatography was performed at room temperature. The resuspended fraction from amicon filtration (approx. volume of 5 ml) was applied to a Q-sepharose column (20 cm x 1 cm), equilibrated in Tris-HCl buffer (1

immediately by washing with 5 column volumes of the same buffer at a flow rate of 0.8 ml/min. The unbound eluate was assayed for anti-PR1 activity to ensure that the inhibitor did not pass through the column. After

loading, the inhibitory activity was eluted with a salt gradient (Buffer A: 10 mM Tris-HCl;pH 7.O: Buffer B: 10mM Tris-HCl with 1 M NaCl; pH 7.0: 0-100% gradient). Fractions were collected using an automated fraction collector (Pharmacia) and the absorbance at 280 nm of each fraction taken. Each fraction was also assayed for anti-PR1 activity

3.2.3.3. High Performance Liquid Chromatography (HPLC).

Those fractions containing antiPR1 activity were pooled and amicon-filtered and resuspended in MilliQ water. The resuspended inhibitor was then lyophilized in a Savant speedvac concentrator and the pellet resupended in a known amount of MilliQ water. This fraction was stored at -20^{o}C until used.

The inhibitor was then subjected to reverse phase high performance liquid chromatography (HPLC) on the system described in section 3.3.3.3.

High performance reverse-phase chromatography used a C_{18}-Dynamax column (Rainin Instruments) with a guard column. The sample was loaded in milli-Q water (Millipore) and eluted with a linear gradient of acetonitrile (0-60% v/v water) at a constant flow rate of 0.75 ml per minute. Both mobile phases were degassed with nitrogen prior to use. Fractions were collected as peaks became visible using a programmable Gilson 201-202 fraction collector.

All fractions were freeze-dried in a Savant speed vacuum. After lyophilisation, the fractions were resuspended in 100 µl 0.1M Tris-HCl; pH 8.0 and tested for inhibitory activity.

3.2.3.4. Gel Filtration Chromatography.

The pure inhibitor was subjected to gel filtration (size exclusion) on a sepharose S-200HR column (100 x 1.6cm) equilibrated with 0.1M Tris-HCl (pH 8.0) and run at a flow rate of 1.1 ml/min. Eluant protein content was determined by reading the absorbance of each fraction at 280nm. The inhibitory activity of each fraction (3.26 ml) was recorded. The molecular weight of the inhibitor was determined from the column after calibration using a series of standards, all at a concentration of 1mg/ml 0.1M Tris HCl. The standards were: cytochrome C (12.4 kDA.), carbonic anhydrase (29.0 kDa.), bovine serum albumen (66.4 kDa.) and alcohol dehydrogenase (150 kDa.)

3.2.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE on a mini-protean II system (Bio-Rad) was carried out to determine the number of protein bands in fractions obtained from chromatography. Polyacrylamide slab gels measuring 100 x 60 mm were cast from a mixture containing 2.5 ml stock acrylamide (Protogel, National Diagnostics, Manville, New Jersey); 2.5 ml separating gel buffer (1.5M Tris-HCl; w/v 0.4% SDS; pH 8.8), 4.85 distilled water, 0.03 ml ammonium persulphate and 0.01 ml TEMED. When polymerization was complete, a stacking gel with castellated wells was cast on top of the resolving gel from a mixture containing 0.4 ml stock acrylamide, 1.2 ml stacking gel buffer (0.5M Tris-HCl; w/v 0.4% SDS; pH 6.8), 0.9 ml distilled water, 0.25 ml ammonium persulphate and 0.005 ml TEMED.

To prepare samples for electrophoresis, those fractions containing the desired activity were lyophilized overnight in a Savant Speedvac

concentrator (Savant, Farmingdale, New York). To remove salt, the pellet was washed in 1 ml 10% ice cold trichloroacetic acid (TCA) for 10 min. The TCA-precipitate was washed 3 times in ethanol-diethylether (50:50 v/v). The resulting pellet was lyophilized for 20 min to remove solvent and resuspended in sample buffer (0.0625M Tris; w/v 2% SDS; 5% mercaptoethanol; 20% glycerol; 0.001% bromophenol blue; pH 6.6) and boiled for 3 min. Ten µl samples were loaded onto the gel which was immersed in electrophoresis buffer (0.05M tris; 0.384M glycine; w/v 0.1% SDS; pH 8.0). Electrophoresis was performed at room temperature at a constant voltage of 200V (30V/cm). Once the tracking dye had reached the bottom of the resolving gel, the gels were removed and stained. Staining of the gels was done by incubating gels in Coomassie blue stain (20% methanol; 10% acetic acid; 0.1% Coomassie brilliant blue R-250) for 15 min at room temperature. protein bands were visualised by putting the gels in destain (25% methanol; 10% acetic acid) for 12 h or until the bands were visible.

3.2.6. Activity PAGE analysis.

In order to visualise inhibitory activity in whole blood, the electrophoretic method of Aratake *et al.* (1990) was employed using some modifications. Electrophoresis was performed on slab gels as before. Samples, however, were not denatured with TCA. Samples were dialysed overnight against water to remove salt and then freeze-dried as before. The samples were then resuspended in non-denaturing sample buffer (0.0625M Tris; w/v 2% SDS; 20% glycerol; 0.001% bromophenol blue; pH 6.6). After electrophoresis, gels were washed in Tris-HCl buffer (10 mM; pH 7.0) containing 1% (w/v) Triton X-100 for 30 min. This was repeated twice before incubating gels in PR1 solution (4mg PR1/100 ml 0.1 M

phosphate buffer; pH 7.4) for 30 min at 37^{o}C. The gels were allowed to stand for 10 min at room temperature before being immersed in staining solution (1mg/ml N-acetyl-D, L-phenylalanine-betanaphthyl ester, 2 mg/ml N,N-dimethylformamide, 10 mM Na_2HPO_4 and 1 mg/ml o-dianisidine tetrazotized).

3.2.7. Protein determination.

For purification steps, total protein was determined by the Bradford Method (Bradford, 1976) using a protein assay kit (Bio-Rad, U.K.). A standard curve was generated each time using a stock solution of bovine gamma globulin. The absorbance of samples was measured at 595 nm.

For cuticle digestion studies, the absorbance at 280 nm and 260 nm were taken and the protein concentration calculated using the expression

$$(0.76 \times A260) - (1.55 \times A280)$$

Harris and Angal, 1989).

3.2.8. Anti-protease activity.

Anti-Pr 1 activity was determined spectrophotometrically in a solution containing Succinyl-Ala-Ala-Pro-Phe-nitroanilide. Fifty µl of PR1 solution (0.1 mg/ml) was incubated for 30 min at room temperature with 400 µl of diluted test sample. This time was used to ensure that any inhibition went to completion. The reaction mixture was made up to 950 µl with 0.1 m Tris-HCl buffer (pH 8.0) and the assay was initiated by the addition of 50 µl of 1 mM substrate. The percentage inhibition was calculated from the expression

$$\% = (1-A_I/A_0) \times 100$$

where A_I and A_O represent the absorbance in the presence and absence of inhibitor respectively.

This approach was employed for determining the amount of inhibitory activity towards other enzymes. The inhibitor was also tested against the following proteases; Chymotrypsin, Proteinase K, Trypsin, Moulting fluid protease 1 (MFP-1; Samuels *et al.*, 1993), Pr2, Subtilisin and Papain. The enzymes were pre-incubated with the inhibitor as above and assayed for inhibitory activity against their respective synthetic substrates (all at 1 mM concentration). These were as follows: Chymotrypsin, proteinase K-Succinyl-Ala-Ala-Pro-Phe-pNA; Trypsin, MFP-1, Pr2- Benzoyl-Phe-Val-Arg-pNA; Subtilisin-Benzoxycarbonyl-Ala-Ala-Leu-pNA; Papain- Benzoyl-Arg-pNA.

3.2.9. Cuticle digestion assay.

Ground [^{3}H]-*Manduca* pupal cuticle was prepared as described in section 4.2.2.2. Cuticle digestion assays were performed by first pre-washing [^{3}H]-*Manduca* pupal cuticle in 10 mM Tris-HCl, 5mM $CaCl_2$, pH 8.0 for 1h. This buffer was removed and fresh buffer added prior to addition of samples. The final assay contained 3 mg of ground cuticle suspended in 50 µl sample and 950 µl buffer. Incubations performed were a) PR1 alone, b) buffer alone, c) PR1 preincubated for 20 min on ice with protease inhibitor and d) protease inhibitor alone. The samples were incubated at 28°C on a cyclogyrator for 6h. Following this they were centrifuged (13000 rpm; 3 min, room temperature) and the supernatant removed. One hundred µl aliquots of sample were then added to 5 ml

liquid scintillant (Optiphase Safe; LKB) and counted using a liquid scintillation spectrophotometer in a LKB Rackbeta counter. Quenching was automatically corrected for by use of an external standard.

Cuticle digestion assays using ground adult *S. gregaria* abdominal cuticle (see section 4.2.2.1.) were also performed. The procedure was the same as above except that the absorbance of the supernatant at 280 nm and 260 nm was taken and the protein content calculated using the expression given in section 4.2.7.

3.3. Results

3.3.1. Identification and partial purification of a protease inhibitor from the blood of *S. gregaria.*

Preliminary experiments suggested the presence of inhibitor(s) of PR1 in the blood of *S. gregaria.* The majority of protease inhibitory activity was in the plasma fraction of the blood (table 16). There was a significant increase in the titre of the inhibitor in the blood of locusts following infection with *M. flavoviride* (330189; figure 17).

The plasma fraction was electrophoresed under non-denaturing conditions and an activity stain was used to determine the position of the inhibitor (figure 18). Inhibitory activity was visualised as a single zone of clearing on an otherwise maroon gel. In order to purify the inhibitory protein(s), 4 ml of blood was pooled and mixed with an equal volume of buffer. After initial extraction (see materials and methods), the sample was passed down an anion exchange column. Figure 19 shows the elution profile of the fractionation and suggests that there may be at least two inhibitors present in the plasma. However, the first peak of activity showed 50% inhibition of PR1 whereas the second peak showed 100% inhibition. The fractions in the latter peak were pooled and passed through a C18 reverse phase column on HPLC. The chromatogram from this step is shown in figure 20. The peak containing the inhibitor was very small (only 0.86% of the protein loaded onto the column) and difficult to obtain without contamination from adjacent peaks. Rechromatography of the collected peak showed barely any protein which could not be detected with silver staining of a SDS-PAGE gel. None of the other peaks from figure 20 showed inhibitory activity against PR1.

Table 16. The distribution of Pr1 inhibitory activity in *S. gregaria* haemolymph[a]

Fraction	% inhibition[b]
Plasma	84.51 ± 8.80
Haemocyte lysate	17.11 ± 5.10

[a] 50 µl blood was collected and mixed with 50 µl anticoagulant buffer and centrifuged at 13000 rpm at 4°C for 4 min. The pellet comprised of haemocytes and the supernatant was plasma. The haemocytes were resuspended in distilled water and recentrifuged. The supernatant was checked for cellular debris. This was the haemocyte lysate.
[b] Percentage inhibition was determined against a standard 0.1 mg/ml solution of Pr1. n=6

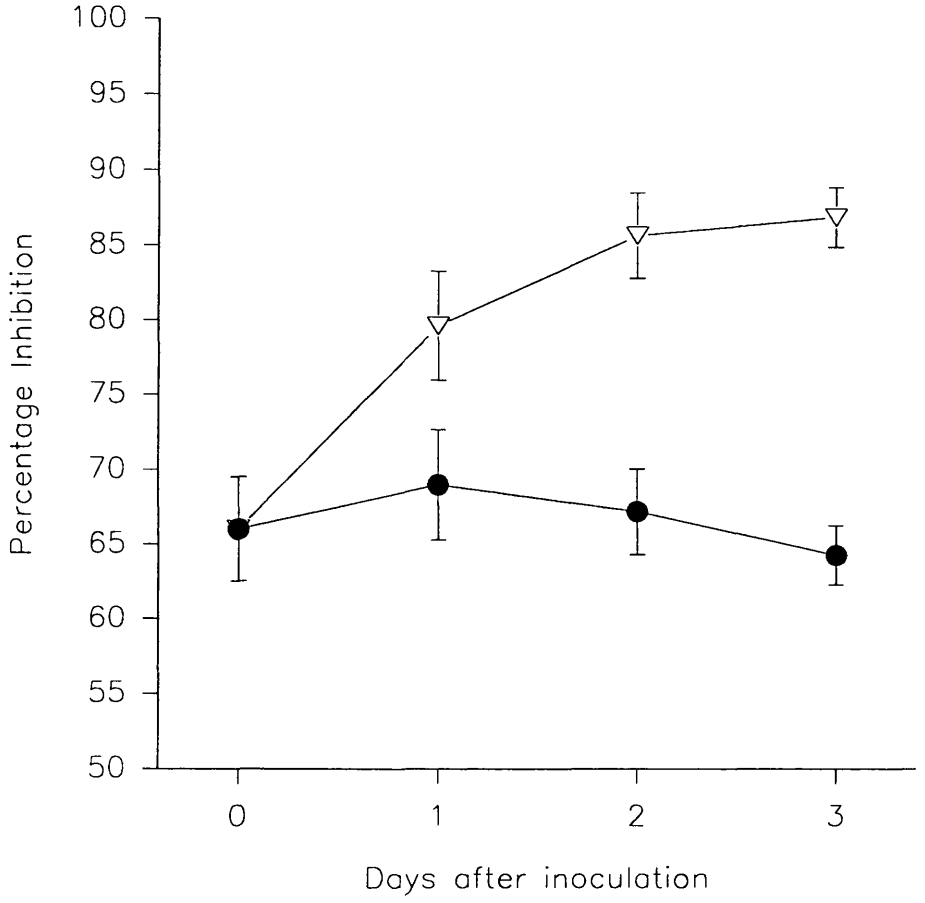


Figure 17. The variation in inhibitory activity against PR1 protease with time after infection with *M. flavoviride* (●) control insects; (▽) infected insects. Values indicate mean plus/minus S.E. (n=12). Difference between means was significant from days 1–3 (Student's *t* test)

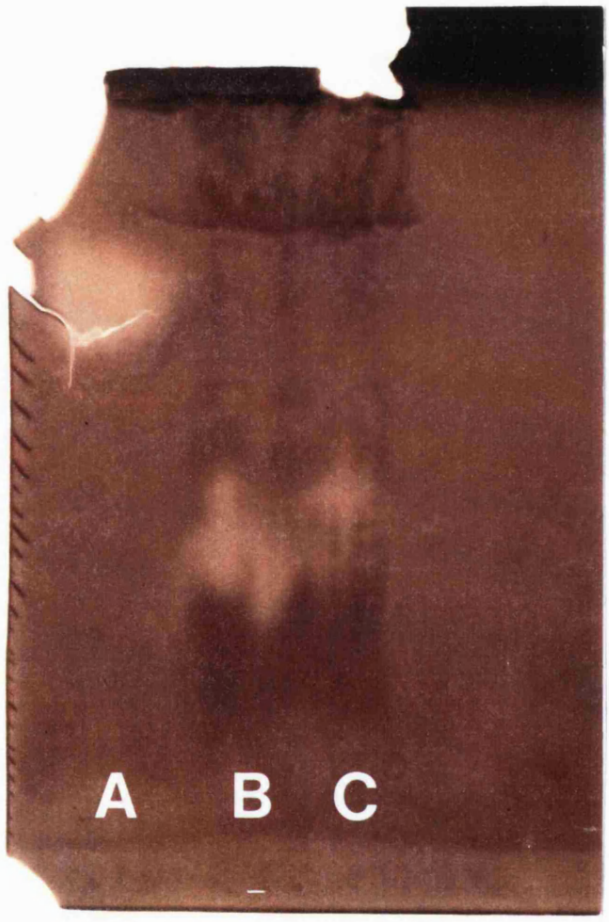


Figure 18: A zymogram to show the localisation of a protease inhibitor in the plasma of *S. gregaria* haemolymph. A; 20 µl of a 0.1 mg/ml solution of turkey egg white inhibitor: B,C; 40 µl of undilute locust plasma.

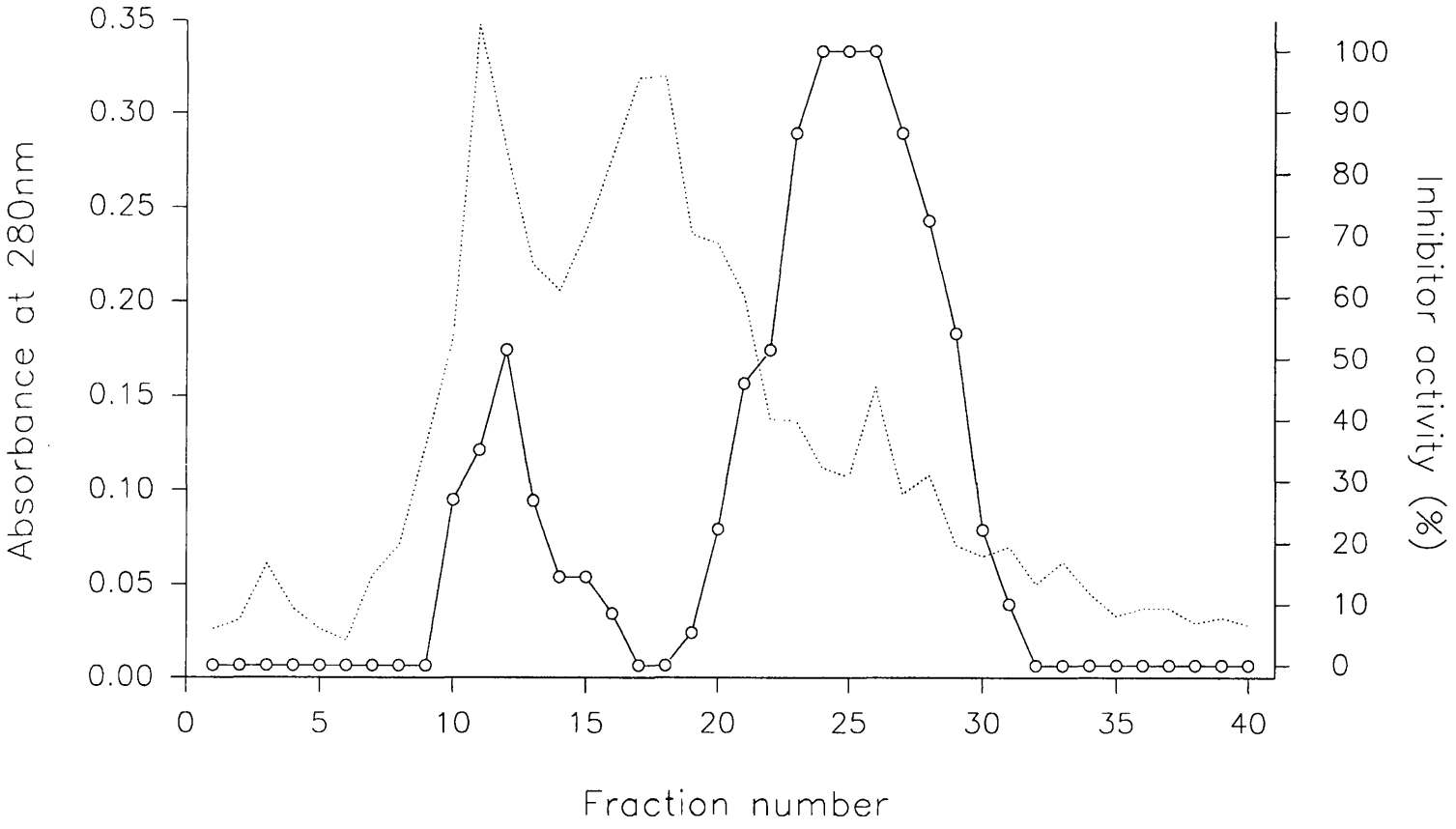


Figure 19. Elution profile of inhibitory activity against the PR1 protease found in the haemolymph plasma of *S. gregaria*. The protein was recovered with a 0.1M NaCl gradient. (·····) Absorbance at 280nm; (o) Inhibitor activity

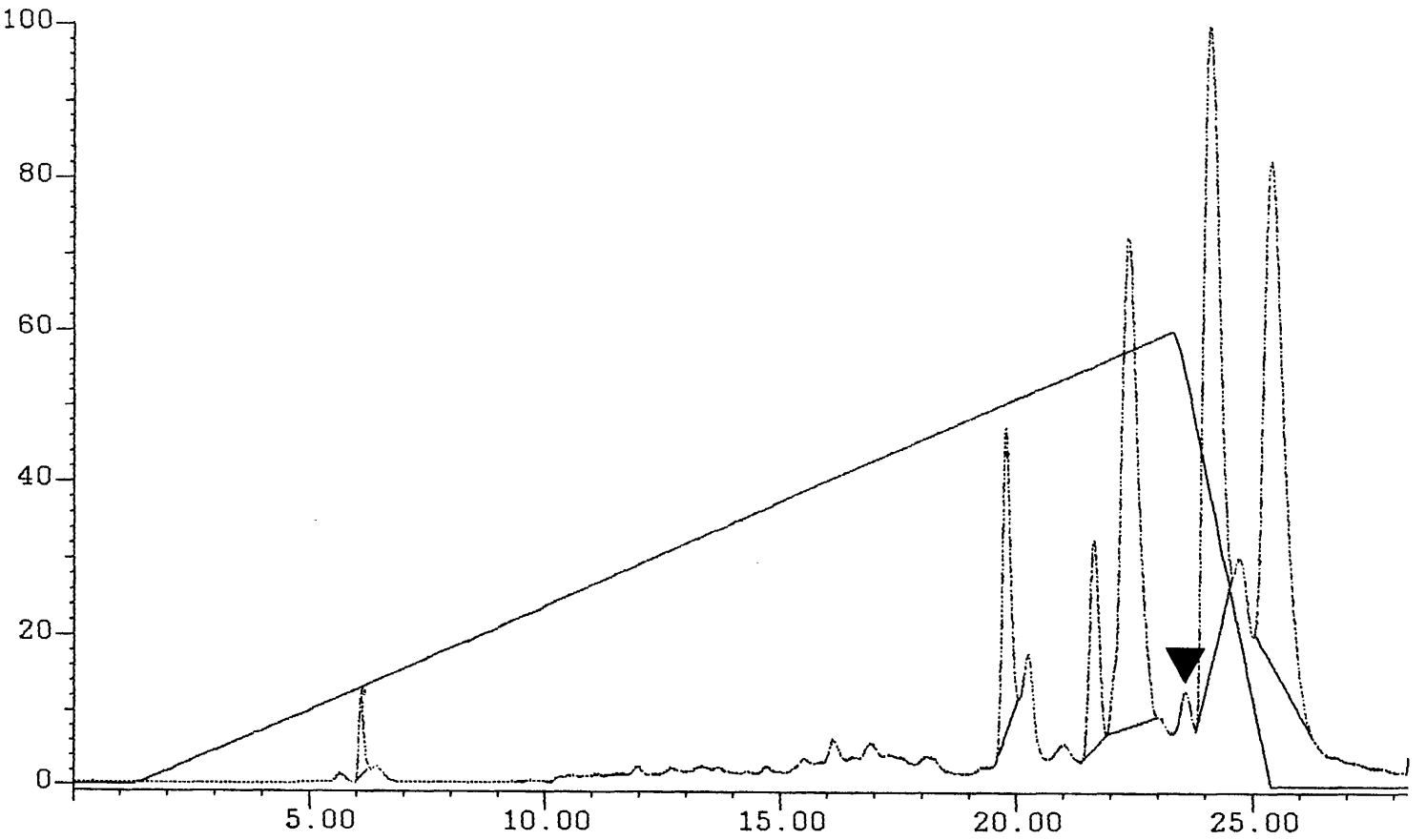


Figure 20: HPLC chromatagram of a protease inhibitor (arrowed) isolated from the plasma fraction of *S. gregaria* haemolymph. Samples were run on a C_{18} dynamax column equilibrated with MilliQ water. The inhibitor was eluted with a gradient of 0-60% (v/v) acetonitrile.

Material purified by anion exchange chromatography was used to investigate the spectrum of inhibitory activity (table 17). The protein was a very good inhibitor of Chymotrypsin, a protease isolated from the moulting fluid of *M. sexta* (MFP-1; Samuels *et al.*, 1993) and proteinase K. Conversely. trypsin was only partially inhibited and Pr2, a trypsin-like enzyme from *M. anisopliae* was uaffected as were subtilisin and papain.

The inhibition of PR1 has thus far been tested against a synthetic substrate. The natural substrate for PR1 is cuticle and the inhibition of digestion was investigated (table 18). The amount of cuticle digested in the presence of inhibitor was greatly reduced (>90%) when compared to that digested in the absence of the inhibitor.

3.3.2. Identification and partial purification of a protease inhibitor from the blood of *M. sexta*.

Preliminary experiments showed that blood plasma from larval *M. sexta* had inhibitory activity against PR1. The anti-PR1 activity was purified to determine whether it was an, as yet, undescribed protein or one of those reported by Kanost (1990) or Ramesh *et al.* (1988). All the inhibitor(s) present in the blood adsorbed to a cation exchanger (figure 21a) and eluted from the column over a large number of fractions (figure 21b). The fractions showing inhibitory activity were pooled and applied to a reverse phase column on HPLC and figure 22 shows the elution profile. One peak showed 100% inhibitory activity to PR1 (Figure 22, arrowed). None of the other peaks showed any inhibitory activity towards PR1, chymotrypsin or trypsin. Rechromatography of the fractions containing the inhibitor showed it to be a single peak with a slight shoulder on the left hand side (figure 23). The eluant was collected after the shoulder had appeared. After confirming the presence of the inhibitor (activity vs. PR1),

Table 17. The effect of partially pure *S. gregaria* protease inhibitor on the activity of a variety of proteases.

Protease[a]	Synthetic substrate[b]	Percentage inhibition[c]
PR1	Succinyl-Ala-Ala-Pro-Phe-pNA	100
Chymotrypsin	Succinyl-Ala-Ala-Pro-Phe-pNA	100
Proteinase K	Succinyl-Ala-Ala-Pro-Phe-pNA	100
Trypsin	Benzoyl-Phe-Val-Arg-pNA	36.2
MFP-1[d]	Benzoyl-Phe-Val-Arg-pNA	100
PR2	Benzoyl-Phe-Val-Arg-pNA	0
Subtilisin	Benzoxycarbonyl-Ala-Ala-Leu-pNA	0
Papain	Benzoyl-Arg-pNA	0

[a] Each enzyme was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) at a concentration of 0.1 mg/ml
[b] Each substrate used was at a final concentration of 0.05 mM.
[c] Values given are the mean of 4 replicates. Standard error was less than 10%
[d] Moulting Fluid Protease (Samuels *et al.*, 1993)

Table 18. The effect of partially pure *S. gregaria* protease inhibitor on the proteolytic activity of purified fungal protease, PR1, isolated from *M. anisopliae* (ME1), on abdominal cuticle

Sample	µg protein released per mg cuticle[a]	percentage inhibition
Buffer + cuticle	0.077 ± 0.008	N.A.
Inhibitor + cuticle	0.063 ± 0.005	N.A.
PR1 + cuticle	0.196 ± 0.004	0
PR1 + cuticle + inhibitor	0.068 ± 0.002	96

[a] Amount of protein determined from A280:A260 values; Values given are for the mean of 7 replicates with standard errors shown.

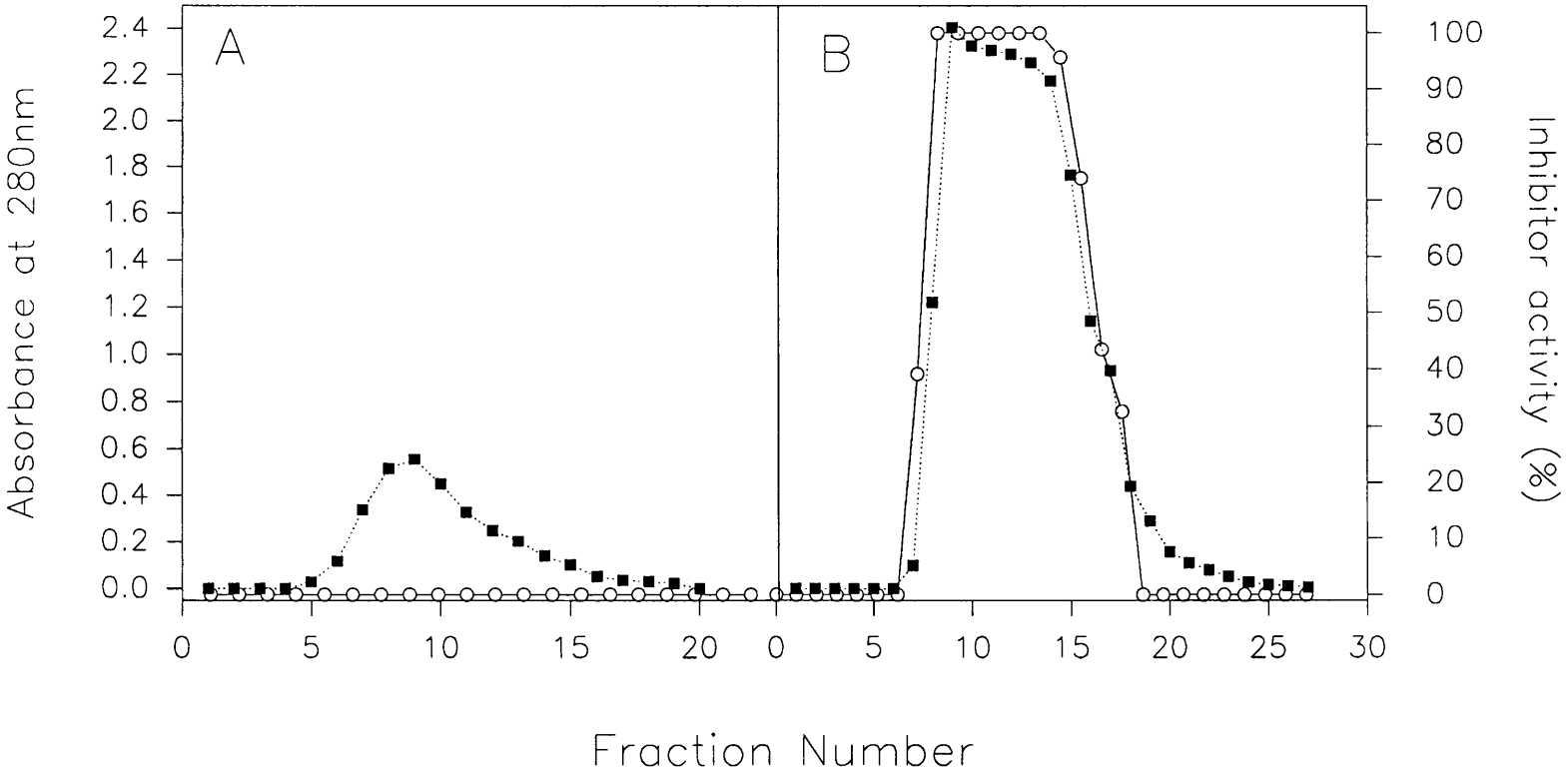


Figure 21. Elution profile of a PR1 inhibitor from the blood of *M. sexta*. A; Unbound protein was allowed to run through a Q-sepharose column. B; bound inhibitor was eluted from the column with 1.0M NaCl. (o) Pr1 inhibitory activity; (■) A280nm

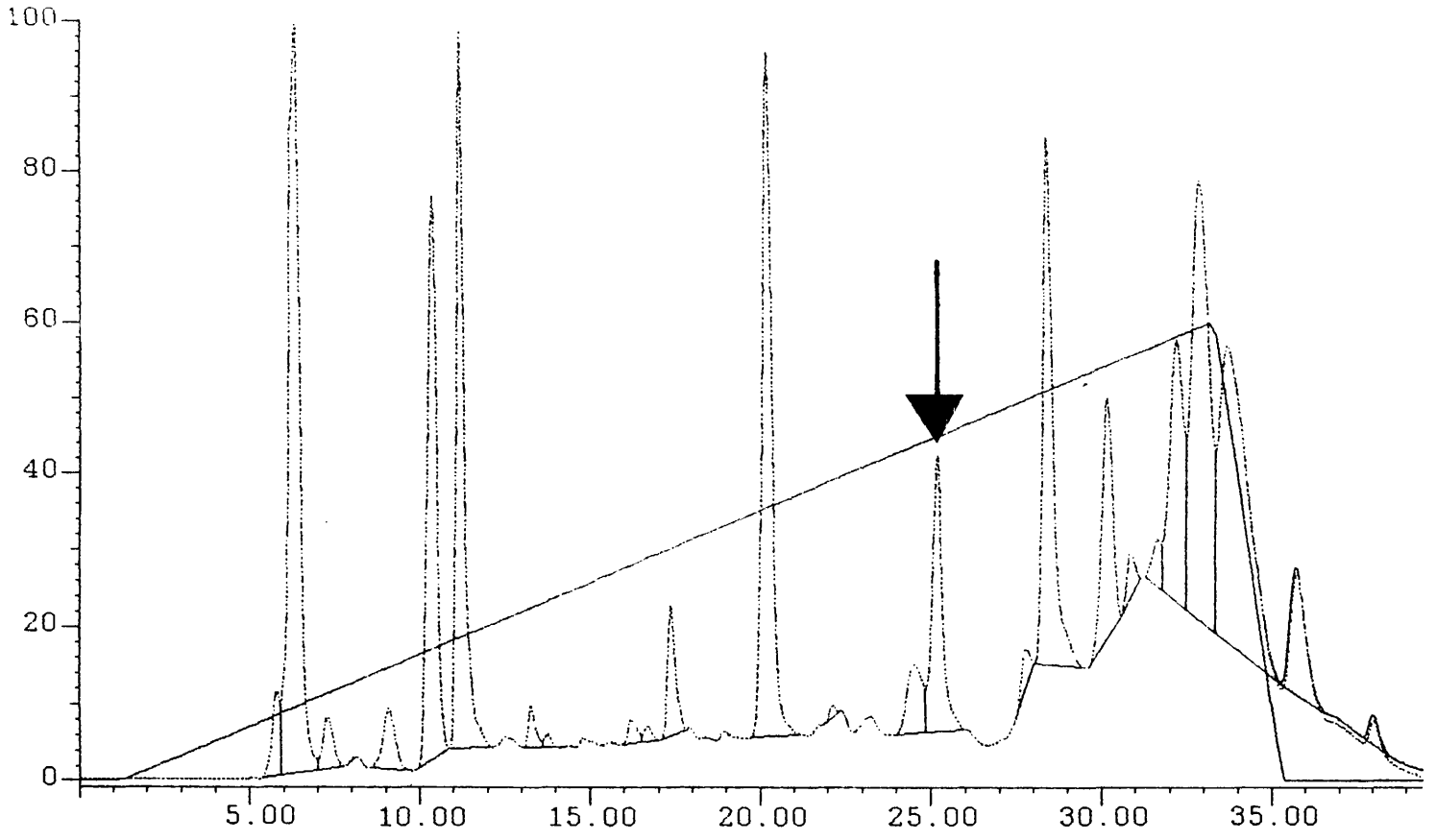


Figure 23: HPLC chromatagram of a protease inhibitor (arrowed) isolated from the plasma fraction of *M. sexta* haemolymph. Samples were run on a C_{18} dynamax column equilibrated with MilliQ water. The inhibitor was eluted with a gradient of 0-60% (v/v) acetonitrile.

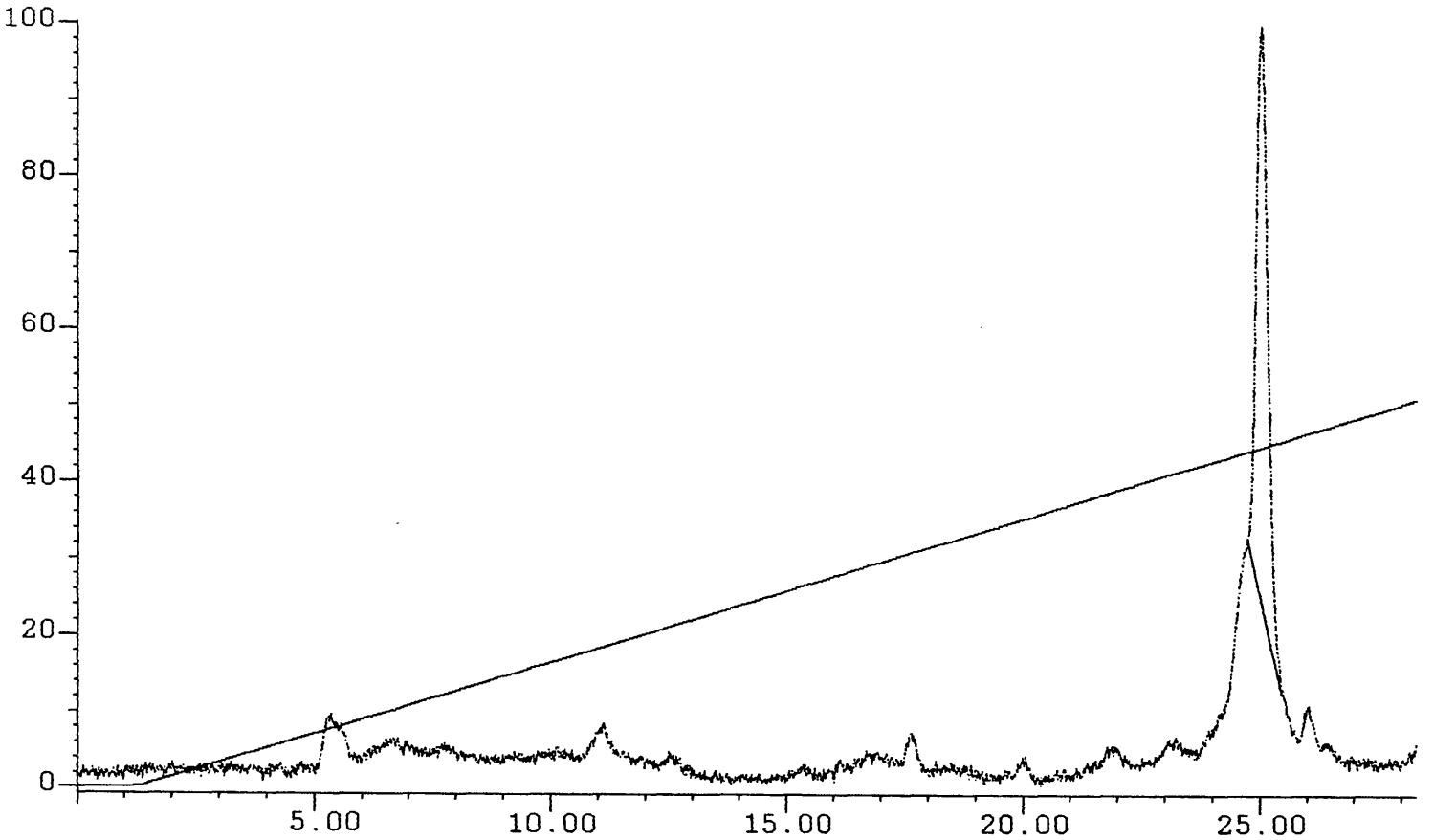


Figure 24: HPLC chromatogram of a protease inhibitor from the plasma of *M. sexta*. The peak shown is the inhibitor identified in figure 6.

the fraction was run on a SDS-PAGE gel. A single protein band was observed which had a molecular weight of 18.2 kDa. (figure 24). Isoelectric focusing confirmed that a single protein (pI 4.5) was present in the fraction (data not shown). Table 19 summarises the purification of the inhibitor. A 12% yield of the protein was obtained with a purification of 102-fold. Gel filtration of the protein confirmed its molecular weight indicating that it is a monomer (figure 25).

It was not possible to obtain enough pure inhibitor for characterisation. Therefore, the properties of the inhibitor were determined using semi-pure preparations viz after the ion exchange step. These preparations contained inhibitory activity against chymotrypsin or trypsin. Table 20 shows the effect of partially pure protease inhibitor on the activity of a range of proteases. PR1 and MFP-1 were inhibited completely. Chymotrypsin and trypsin were only partially inhibited whilst proteinase K, Pr2, subtilisin and papain were unaffected. The inhibitor completely prevented hydrolysis of a preparation of ^{3}H-pupal cuticle from *M. sexta* by PR1 (table 21).

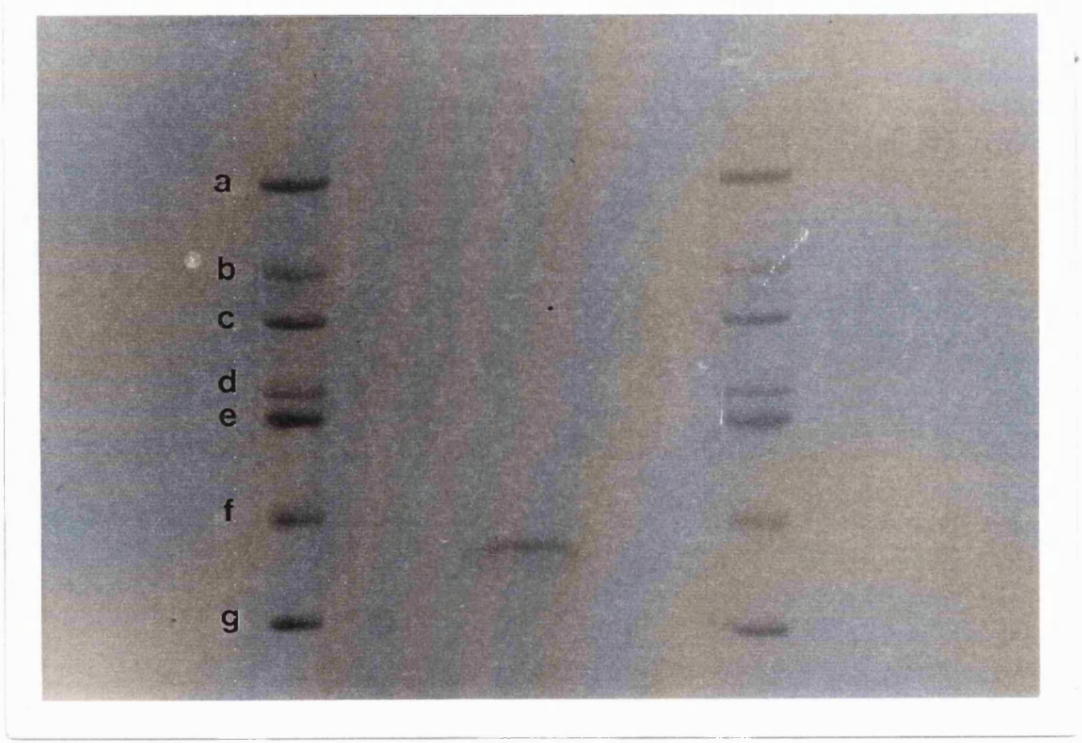


Figure 25: SDS-PAGE of a protease inhibitor isolated from the plasma of *M. sexta* haemolymph and stained with Coomasie Blue. Molecular markers are shown: a; lactalbumin (14.2 kDa.): b; soybean trypsin inhibitor (20.1 kDa.): c; trypsinogen (24.0 kDa.): d; carbonic anhydrase (29.0 kDa.): e; glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa.): f; egg albumin (45.0 kDa.): f; bovine albumin (66.0 kDa.).

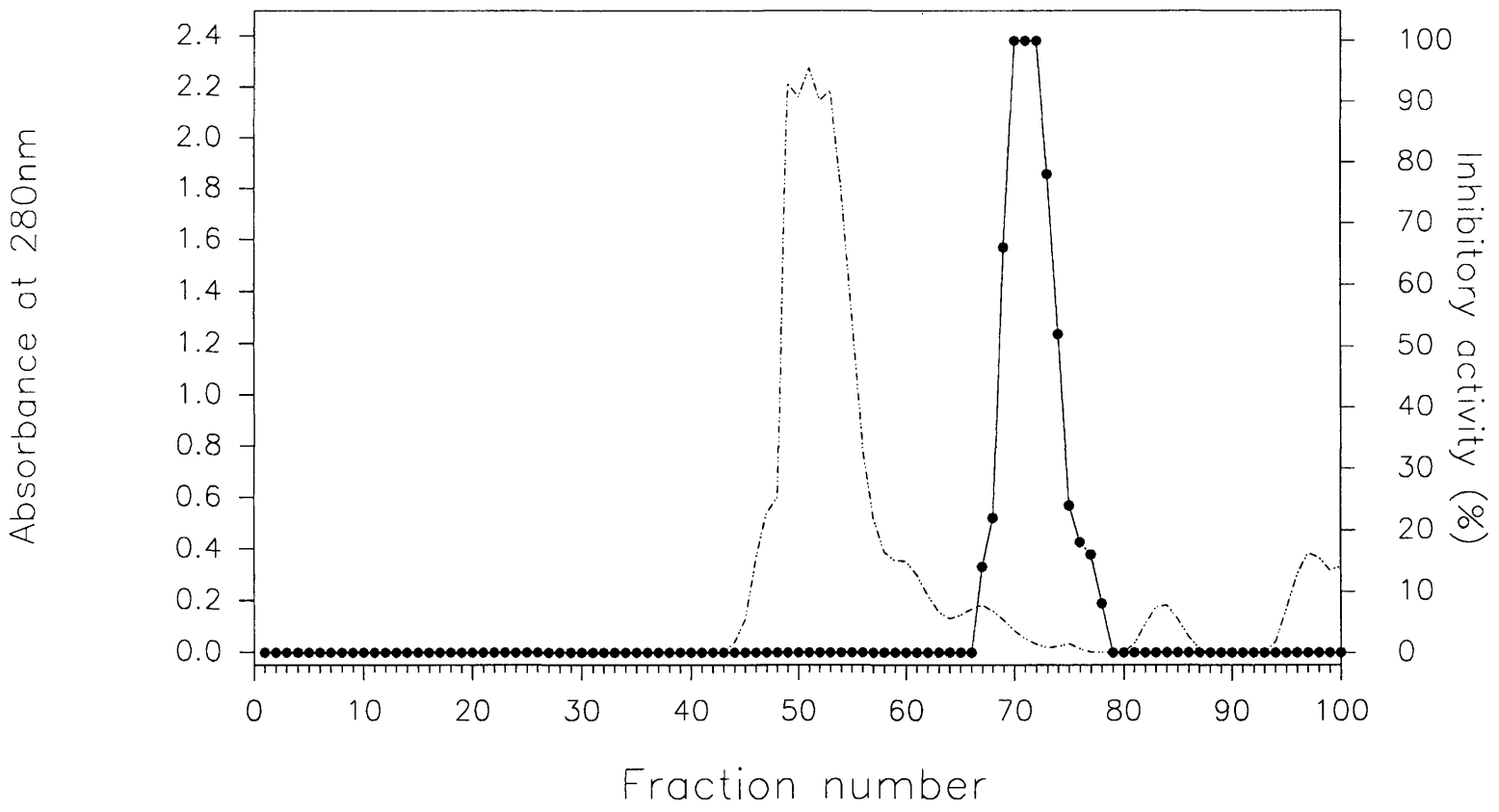


Figure 25. Gel filtration profile of a novel protease inhibitor from the blood of *M. sexta*. The protein was passed down the column in 0.02M NaCitrate buffer. (•) protease inhibitory activity; (------) Absorbance at 280nm

Table 19. Summary of purification of the PR1 protease inhibitor from *M. sexta* haemolymph.

Step	Total volume (ml)	Total activity (Units)	Total Protein (mg)	Specific activity (Units/mg protein)	Yield (%)	Fold Purification
Centrifuged haemolymph	21	10500	257.9	40.71	-	-
Ammonium sulphate precipitation	30	3000	211.8	14.16	100	0.49
Q-sepharose chromatography	15.88	1588	60.7	26.16	52.9	0.29
Reverse-phase chromatography	3	360	0.25	1440	12.0	101.69

Table 20. The effect of partially pure *M. sexta* PR1 protease inhibitor on the activity of a variety of proteases.

Protease[a]	Synthetic substrate[b]	Percentage inhibition[c]
PR1	Succinyl-Ala-Ala-Pro-Phe-pNA	100
Chymotrypsin	Succinyl-Ala-Ala-Pro-Phe-pNA	84.5
Proteinase K	Succinyl-Ala-Ala-Pro-Phe-pNA	0
Trypsin	Benzoyl-Phe-Val-Arg-pNA	67.3
MFP-1[d]	Benzoyl-Phe-Val-Arg-pNA	100
PR2	Benzoyl-Phe-Val-Arg-pNA	0
Subtilisin	Benzoxycarbonyl-Ala-Ala-Leu-pNA	0
Papain	Benzoyl-Arg-pNA	0

[a] Each enzyme was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) at a concentration of 0.1 mg/ml
[b] Each substrate used was at a final concentration of 0.05 mM.
[c] Values given are the mean of 4 replicates. Standard error was less than 10%
[d] Moulting Fluid Protease (Samuels *et al.*, 1993)

Table 21. The effect of *M.sexta* protease inhibitor on the proteolytic activity of purified fungal protease, PR1, isolated from *M. anisopliae* (ME1), on [^{3}H]-cuticle

Sample	DPM[a]	percentage inhibition
Buffer + cuticle	168.5 ± 26.0	N.A.
Inhibitor + cuticle	169.4 ± 11.1	N.A.
PR1 + cuticle	1157.7 ± 215.8	0
PR1 + cuticle + inhibitor	135.7 ± 14.1	100

[a] Disintegrations per minute; Values given are for the mean of 7 replicates with standard errors shown.

3.4. Discussion

A protease inhibitor from the haemolymph of *M.sexta* has been purified to homogeneity by ion exchange and reverse phase HPLC chromatography. It is a monomer with a molecular weight of 18.2 kDa and an isoelectric point of 4.6. Evidence suggests that this is a newly reported inhibitor and not one of those described by Kanost (1990a) or Ramesh *et al.* (1988) and I propose that this inhibitor be called the *Manduca sexta* PR1 inhibitor (MSPRI).

Kanost (1990) isolated four related serine protease inhibitors (serpins) from the blood of *M. sexta.* However, the molecular weight of these proteins was in the range 45 to 47 kDa whilst the pIs were in the region 4.4-4.8. Western blotting of the pure MSPRI using antiserum generated against serpins (a kind gift of Dr. M. Kanost) showed no cross-reactivity (data not shown) providing evidence the inhibitor was not a breakdown product of a serpin.

Ramesh *et al* (1988) have identified two Kunitz-type trypsin inhibitors in the blood of *M. sexta.* However, the new inhibitor is different from HLTI A, which is a dimer with a molecular weight of 14 kDa and a pI of 5.7, and HLTI B, which is a monomer of 8 kDa (pI 5.3 and 7.1). Antigenic relatedness of these molecules to the new inhibitor could not be determined since no antiserum to these inhibitors was available.

The presence of protease inhibitors in orthopteran insects has, until recently, not been reported. Polanowski *et al.* (1992) observed the mild inhibition of chymotrypsin and cathepsin G by blood from the grasshoppers, *Romalea guttata* and *Taeniopeda eques* and strong inhibition of cathepsin G and elastase with the weak inhibition of chymotrypsin were observed in the cricket, *Acheta domestica.* However, their observations were based on spectrophotometric assays using crude blood preparations.

Boigregain *et al.* (1992) have isolated two low molecular weight protease inhibitors from the fat body of *L. migratoria*. The present work is the first demonstration of a protease inhibitor in the blood of the desert locust. Unfortunately, purification of this inhibitor proved difficult and ion exchange chromatography revealed the presence of possibly two inhibitors in desert locust blood.

The natures of the inhibitors from both *Manduca* and *Schistocerca* remain unknown. Both inhibitors prevented PR1 activity against anilide substrates and insect cuticle indicating that they were not α_2-macroglobulin-like. This is not surprising as these type of inhibitors have only been previously reported in marine arthropods and are all large molecules (>100 kDa).

The protease inhibitors from the desert locust and the tobacco hornworm had broadly similar spectra of activity. The key exceptions were that the former inhibited protease K and had limited activity against trypsin (36%) whereas the latter had no effect on protease K and substantial activity against trypsin (67%). Both inhibited PR1, chymotrypsin and MFP-1 but not PR2, subtilisin and papain.

Both *M. sexta* (present work; Kanost, 1990a; Jiang *et al.*, 1994) and *B. mori* (see Eguchi, 1993) produce a number of protease inhibitors. This may reflect the variety of roles that the inhibitors play, but also may reflect diversity among pathogen proteases. Multiple isoforms of PR1 and PR2, a metalloprotease and a trypsin-like cysteine protease (PR4) have all been found in culture filtrates of *M. anisopliae* (see Charnley and St. Leger, 1991; Cole *et al.*, 1993; St. Leger *et al.*, 1994a).

The PR1 inhibitors from locust and tobacco hornworm may have more than one function. Both inhibitors were found to inhibit the activity of the moulting fluid protease, MFP-1 against anilide substrate. MFP-1, a protein of 41 kDa molecular weight with a pI of 5.54, has recently been

purified in this laboratory (Samuels *et al.*, 1993). It is a trypsin-like enzyme that degrades *Manduca* cuticle *in vivo*. One possible role for the inhibitor could be to prevent hydrolysis of host proteins if moulting fluid proteases accidentally leak into the haemocoel. Samuels has identified protease inhibitors in the moulting fluid of *M.sexta* (Reynolds, Pers. Comm.). One was purified to a single peak on reverse phase HPLC and gel filtration HPLC indicated that it had a molecular weight of around 2.5 kDa. It inhibited chymotrypsin, tryspin, proteinase K, MFP-1 and PR1 but not PR2. Furthermore, it was present early in the moulting process but disappeared just before ecdysis. The low molecular weight of this inhibitor indicates that it is not the same as the one identified here.

Inhibitors from both locusts and tobacco hornworms prevent the hydrolysis of cuticle by PR1 *in vitro*. This suggests that the inhibitors may additionally contribute to host defences against fungal pathogens. Numerous inhibitors have been found in the larval blood from the lepidopterans *B. mori* and *M. sexta* (see Eguchi, 1993) and one in insect cuticle (Yoshida *et al.*, 199o). Phenoloxidase has been found in both the blood and the integument and Kanost (1990a) suggested the function of blood borne protease inhibitors may be prevent undesired prophenoloxidase activation. Therefore, it is perhaps to be expected that protease inhibitors should be present in the cuticle as they are in the moulting fluid.It is known that protease inhibitors from crayfish and *M. sexta* can regulate the activation of prophenoloxidase (Saul and Sugumaran, 1986, Hall and Soderhall, 1982). The involvement of MSPRI in pPO regulation was not determined.

Another possible function for these inhibitors may be to prevent the action of proteases leaked into the haemocoel from the insect gut. The serpins from *M. sexta* have been shown to inhibit the midgut trypsin-like

enzymes (Kanost, Pers. comm.). The inhibitors isolated here have not been tested against the midgut proteases from any source.

In summary, inhibitors with potent inhibitory activity toward the fungal cuticle-degrading enzyme, PR1 have been identified in the plasma of *M. sexta* and *S. gregaria* but only the former inhibitor has been successfully purified. Whilst some of the characteristics of the *Manduca* inhibitor have been identified, its specific function remains unknown. The sequence and kinetic properties of this inhibitor are needed if its major function and relatedness to other protease inhibitors are to be determined.

Chapter 4

4.0. The role for cuticle degrading proteases in the pathogenicity of *Metarhizium* spp. for the desert locust, *S. gregaria*.

4.1. Introduction.

Insects possess an external skeleton or cuticle which, apart from functioning as a muscular support, prevents desiccation of the host and invasion of many potentially lethal micro-organisms. Unlike entomopathogenic bacteria and viruses which invade host insects actively through the alimentary canal, the entomopathogenic fungi infect by penetrating the host cuticle.

Fungal penetration of insect cuticle is facilitated by a combination of mechanical force and enzymatic degradation (Goettel *et al.*, 1989; Zacharuk, 1970). The relative contribution of each is dependent upon the structure and composition of the cuticle encountered (Charnley, 1984).

Following adhesion to the surface of the host cuticle, the fungal spore germinates. However, invasion of the cuticle often requires the formation of specialised infection structures such as appressoria and penetration pegs. Whereas germination of *M. anisopliae* occurs in response to non-specific carbon and/or nitrogen sources (St Leger *et al.*, 1986a), differentiation has specific requirements. It has been shown that low levels of complex nitrogenous compounds induce appressorial formation against hard hydrophobic surfaces. However, for most isolates, higher nutrient levels suppress infection-related morphogenesis and cuticular penetration Instead, germ tubes elongate and branch forming a hyphal mat (St. Leger *et al.*, 1989).

Most entomopathogenic fungi invade the host to obtain nutrients for growth and reproduction. Penetration can occur via wounds (see St Leger, 1991), sense organs (McCauley *et al.*, 1978), tracheae (Hedlund and Pass, 1968) and most commonly by direct penetration of the cuticle (Charnley, 1984; 1989; Goettel *et al.*, 1989). Invasion via cuticular pore canals is rarely reported (David, 1967) whilst the alimentary canal is generally an unfavourable environment for fungi (Dillon and Charnley, 1991).

4.2. The Cuticle as Barrier to Infection.

Insect cuticle is a laminate structure that may be divided into two main zones, the epi- and pro-cuticles (figure 26).

The epicuticle is the first barrier encountered by a fungal pathogen. It is a very thin composite structure which contains no chitin but contains phenol-stabilised (sclerotized) protein (Andersen, 1991). It is a multilayered structure with each layer having individual properties. The outer cuticulin envelope contains proteins that undergo sclerotization (Locke, 1984). This outer layer appears to be fragile (Hackman, 1984) and may be penetrated by a weak mechanical force such as that provided by a penetration peg (St Leger, 1991). Furthermore, its resistance to enzymatic degradation indicates that, unless disrupted mechanically, it could prevent the progress of fungal enzymes. The inner epicuticle is a physically tough layer thought to consist of lipoprotein stabilised by quinones (Dennell, 1946). The epicuticular proteins tend to be inextractable and quantitatively they constitute a very minor part of the total cuticular protein.

The procuticle contains chitin fibrils embedded in a protein matrix, together with lipids and, in some cases, quinones and polyphenols (Hepburn, 1985). This thick layer presents the fungus with a formidable

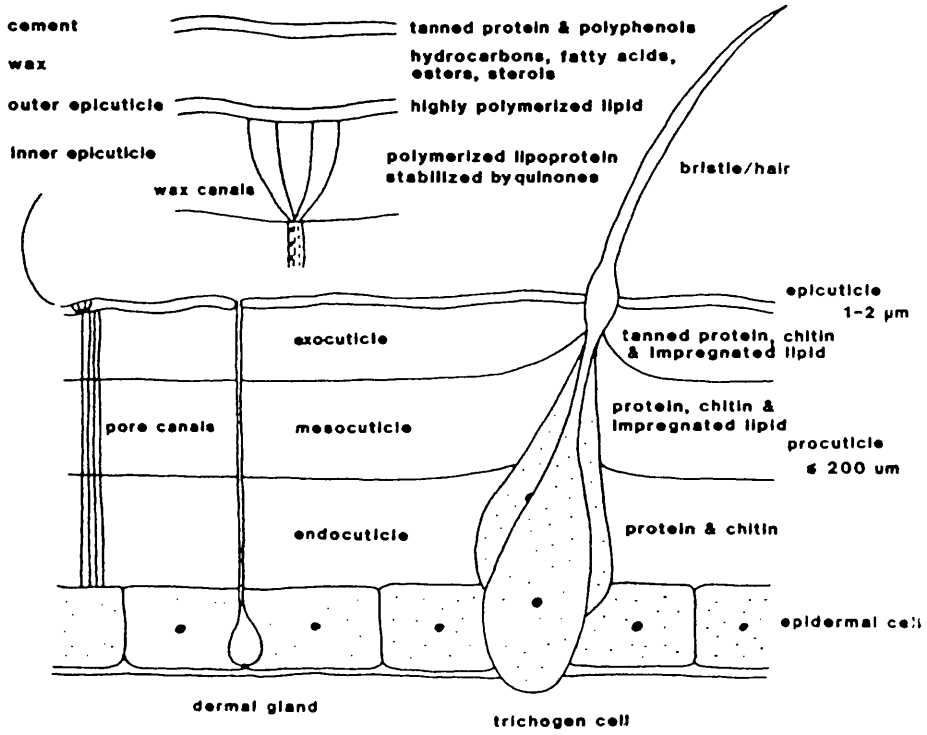


Figure 26. Structure of the insect cuticle (Charnley and St. Leger, 1991).

barrier. In insects such as locusts, the procuticle is subdivided into an outer exocuticle and an inner endocuticle. The exocuticle contains protein that has been sclerotized which could be important in protecting the unsclerotized endocuticle from digestion by the fungal proteases. The mechanical properties of different cuticles depend upon the proportions of the two main constituents, the nature and extent of hydration of the protein and the degree of sclerotisation or tanning (Hillerton, 1984).

4.3. Cuticle Degrading Proteases.

The entomopathogenic fungi produce an array of endo-and exo-acting proteases in culture. Some of the enzymes characterised thus far are the collagenases from *Entomophthora coronata* (Hurion *et al.*, 1977) and *Lagenidium giganteum* (Hurion *et al.*, 1979), the chymoelastases and trypsins produced by a variety of Hyphomycetes including *M. anisopliae* and *B. bassiana* (St. Leger *et al.*, 1987a, c; Bidochka and Khachatourians, 1987b).

The focus of this section will be the proteases produced by *M. anisopliae* as these enzymes have been studied extensively and their role in pathogenesis has been investigated.

4.3.1. Endoproteases.

When culture filtrates of *M. anisopliae* were grown on cuticle, the extracellular proteolytic activity could be resolved into 3 components, PR1 and PR2 with alkaline pH optima and PR3 with acidic pH optima (St. Leger *et al.*, 1987b). Of the three, PR1 was found to be the most effective at degrading cuticle with additional activity against elastin, bovine serum albumin, collagen and casein.

Until recently, PR1 activity was ascribed to a single enzyme and, to date only one PR1 gene has been cloned (St Leger *et al.*, 1992). However, it has since been shown that PR1 occurs as four alkaline hydrolases with a pI range of 9.3-10.2 (St. Leger *et al.*, 1994a). They possess a broad primary specificity for amino acids with a hydrophobic side group at the second carbon atom (i.e. phenylalanine, methionine and alanine). However, they possess different secondary specificities with the original PR1 isoform characterised (pI 10.2; Charnley and St. Leger, 1991) favouring extended hydrophobic peptide chains with the active site recognising at least five subsite residues. Two of the other isozymes prefer bulky hydrophobic amino acids at the P2 and P3 positions. The N-terminal amino acid sequences indicate that the isoforms are related products from two distinct genes. The functional reason for the presence of four types of enzyme has not been determined.

Similar proteases to PR1 with acidic, neutral or acid IEF points have been found in *B. bassiana, V. lecanii, A. aleyrodis* and *N. rileyi* (St. Leger *et al.*, 1987c), and *V. chlamydosporum* (a nematophilic fungus; Segers *et al.*, 1994). The acidic enzymes occur as multiple isozymes which resemble alkaline PR1 in their primary specificity but are unable to degrade elastin (see Charnley and St Leger, 1991). The negative charge of elastin results in the adsorption of alkaline PR1 but not acidic forms. The adsorption of the enzyme to its substrate has been found to be a prerequisite for activity (St Leger *et al.*, 1986b). Similarly, PR1 must bind to negatively charged cuticular groups because, only following adsorption, does the active site of PR1 come into contact with susceptible peptide bonds (St. Leger *et al.*, 1986a). As the cuticular proteins become solubilised by the enzyme, further degradation occurs until a final peptide chain length of 5 is obtained (St Leger *et al.*, 1986c).

A non-serine proteinase with PR1-like activity has also been identified in culture filtrates of *M. anisopliae* (St Leger *et al.*, 1994b). This enzyme was inhibited by a metalloproteinase inhibitor as well a thermolysin inhibitor. The substrate specificity of this enzyme is also consistant with a member of the metalloproteinase family. This enzyme may complement PR1s and PR2s (see below) and/or serve as a back-up to the PR1s which are sensitive to the serine protease inhibitors present in the host blood system and cuticles (St Leger *et al.*, 1994a)

M. anisopliae PR2 occurs as two major isozymes (pI 4.4 and 4.9) with comparatively low activity against insect cuticle but with high activity against casein. The isoform with a pI of 4.4 had a specificity for arginine and lysine residues (similar to bovine trypsin) and was sensitive to trypsin inhibitors such as leupeptin (St. Leger *et al.*, 1987b). St. Leger *et al.* (1987c) suggested that PR2 may be involved in cellular control mechanisms such as the catalysis of specific proteolytic inactivation/activation mechanisms.

A third enzyme, designated PR3, with an apparent pH optimum of 5.0-5.5 has been described but not characterised (St Leger *et al.*, 1987b). Cole *et al.* (1993) have purified a cysteine protease (PR4) with a pI of 5.4 and a molecular mass of 26.7 kDa whilst also possessing trypsin-like substrate specificity. PR4 had a higher cuticle degrading activity than PR2 but not PR1 and its significance in pathogenesis remains to be elucidated.

4.3.2. Exoproteases.

Two classes of aminopeptidase have been isolated from cuticle-grown cultures of *M. anisopliae* and classified as an aminopeptidase M with a broad specificity and a post-prolyl dipeptidyl aminopeptidase IV (St. Leger *et al.*, 1993).

The aminopeptidase (pI 4.51, Mr 45000) exists as multiple isomers with maximal activity for alanyl residues with lesser activity against apolar and hydrophobic amino acids. Inhibition by bestatin and amastatin indicates similarities with the alanyl aminopeptidase (aminopeptidase M) class enzymes. Inhibition by metal complexing agents such as EDTA and 1,10 phenanthroline suggest that it is a metalloprotease.

The dipeptidylpeptidase (aminopeptidase IV; pI 4.01; Mr 74000) exists as two isomers. The enzyme preferentially removes the penultimate prolyl residue from substrates such as alanyl-prolyl-glycine and aa-prolyl-β NA whilst having a broad specificity at the N-terminal amino acid. Inhibition by diisopropylfluorophosphate indicates that the enzyme had a serine residue involved in catalysis. Diprotin A also inhibited the enzyme showing that there are similarities between it and the mammalian aminopeptidases A and M.

Interestingly the two enzymes isolated showed different patterns of production. Whilst the aminopeptidase was produced only on cuticle cultures, the dipeptidylpeptidase was produced on casein or cuticle. Furthermore, there were multiple forms of the latter enzyme produced on casein suggesting that *M. anisopliae* produces these enzymes as an adaptation to different nutrient conditions (St. Leger *et al.*, 1993; St. Leger *et al.*, 1995). The role of these enzymes in parasitism has yet to be defined but they probably provide nutrients for the fungus.

4.4. Regulation of Production.

The regulation of PR1 and PR2 is not identical although both are produced in less than 2 hours in culture under carbon and nitrogen de-repression. In minimal media, the soluble protein bovine serum albumin represses production of PR1 but not PR2. The addition of more readily

utilised metabolites such as glucose or alanine, repressed extracellular protease production confirming that production is constitutive but repressible (St Leger *et al.*, 1988a)

Maximal levels of PR1 were found to be produced in cultures grown on insect cuticle as the sole carbon and nitrogen source (Patterson *et al.*, 1994a). These results were consistent with specific induction of PR1 by insect cuticle. Patterson *et al.* (1994b) attempted to identify the inducers and showed that whilst deproteinised cuticle, chitin or cuticle treated with organic solvent to remove lipids did not induce PR1, peptides hydrolysed from cuticle by pure PR1 and PR2 did. Patterson *et al.* (1994b) suggested that induction is due to oligopeptides released from cuticle by basal levels of PR1 and PR2. Therefore, it seems that some component, yet to be identified, of locust cuticular protein is responsible for inducing PR1.

PR1 is produced during appressorial formation on artificial surfaces or insect cuticle (St Leger *et al.*, 1989b). It is the major protein synthesised during maturation of appressoria and production of penetration pegs. This rapid production of protease could only be possible on host tissues where the concentration of readily utilisable compounds is low. This is the case with insect cuticles where the components are mainly insoluble until released by cuticle degrading enzymes (St Leger *et al.*, 1987b). Repression would therefore exist when the release of degradation products from the cuticle exceeded the nutritional requirements of the fungus. Addition of alanine resulted in extensive growth of fungus on the cuticle but repressed appressorial formation and the production of PR1 (St Leger *et al.* 1989b). Charnley and St. Leger (1991) have shown that the control of enzyme production is at the level of transcription. However, it has not been determined what actually regulates transcription. The promoter region of the PR1 gene form *M. anisopliae* has been sequenced (S.E. Screen, Unpub.) and putative binding sites for regulatory proteins have been

identified. These sites are homologous to the *creA* and *areA* sites found in *Aspergillus* spp. These sites may be involved in carbon and nitrogen derepression (S.E. Screen, Pers. Comm.).

With regards to PR2 production, Patterson *et al.* (1993) have shown that regulation of this enzyme is controlled by multiple regulatory circuits which include carbon and nitrogen metabolite repression as well as induction by a range of proteins.

In summary, it has been shown that PR1 and PR2 are produced constitutively but are also controlled by multiple regulatory circuits which include carbon and nitrogen derepression and induction. It is evident that, under derepressed conditions, PR2 is induced by a range of proteinaceous substances whilst PR1 is induced by an, as yet unidentified, proteinaceous component of insect cuticle.

4.5. The role of endoproteinases in pathogenesis/virulence.

The pathogenicity of an organism is given as the ability of that organism to cause disease or pathological changes (Mims, 1988). On the other hand, virulence refers to a quantitative measure of pathogenicity. For example it can be expressed as the number of organisms necessary to cause death in 50% of individuals, or lethal dose (LD50). A role for PR1 in fungal pathogenesis is indicated by the fact that mortality was retarded in insects inoculated with conidia of *M. anisopliae* that had been simultaneously treated with turkey egg white inhibitor (TEWI) or antibodies raised against the PR1 enzyme (St. Leger *et al.*, 1988b). Furthermore, the hydrolysis of cuticle was significantly reduced when PR1 was inhibited with TEWI. In *B. bassiana*, protease deficient mutants had a reduced LT_{50} towards grasshoppers (Bidochka and Khachatourians, 1990).

Intracellular activities of PR2 exceed those of PR1, the reverse of which is true when looking at extracellular activities (see Charnley and St. Leger, 1991). This would suggest an intracellular role for PR2 such as catalysing specific proteolytic activations. Furthermore, this would account for the observation that PR2 is less susceptible to catabolite repression than PR1. Interestingly, this does not preclude an extracellular role for PR2 as extracellular PR2 in cultures grown on cuticle appears earlier than PR1 and reaches higher activity as well (Kershaw, 1993). This is consistent with the hypothesis that cuticle degradation products from PR2 induce PR1 (Patterson *et al.,* 1994b).

Whilst PR1 appears to have a role in pathogenicity, the relationship between its production and isolate virulence is less clear. Molecular and non-molecular approaches could be employed to investigate this relationship. Molecular techniques include mutation of the promoter region of the gene coding for the most efficient PR1 isozyme causing it to be up-regulated. A corresponding increase in isolate killing power would help to establish a role for the enzyme in virulence. Non-molecular techniques involve, for example, investigating the amount of enzyme produced on cuticle *in vitro* and *in vivo* by a range of isolates and correlating it with virulence. It is known that single amino acid changes in the PR1 sequence can influence activity against cuticle (St. Leger, Pers. Comm.). Thus, it is possible that the activity of PR1 from different isolates will vary slightly in their sequence and ability to degrade insect cuticle and that this may affect virulence. The host cuticle itself may prove to be a factor affecting the efficiency of the enzyme since there are differences between insects in the protein composition of their cuticles (Andersen *et al.*, 1995). Indeed, there are differences in the protein composition of cuticle from different regions of the same insect (see Andersen *et al.*, 1995).

The aim of this study was to correlate the *in vitro* production of PR1 and PR2 with the virulence of 19 isolates of *Metarhizium* spp for adult desert locusts. In addition, this study investigated the ability of purified preparations of PR1 to degrade different types of insect cuticle and attempts were made to correlate the data obtained with virulence. The presence of PR1 in infected insect blood has not previously been investigated in insects infected with *M. anisopliae*. An enzyme linked immunsorbent assay (ELISA) has been developed that can potentially detect PR1 *in vivo*.

4.2. Materials and Methods.

4.2.1. Fungal Isolates.

The isolates of *Metarhizium* spp. used in this study and their origin are listed in Table 22. Isolates were obtained from one of three sources: United States Department of Agriculture collection of fungal cultures (USDA-ARSEF); International Institute of Biological Control (IIBC), Silwood Park, Ascot, U.K. or Horticultural Research International (HRI), Littlehampton, U.K.

4.2.1.1. Maintenance and culture.

Stock cultures were grown on 1/4 strength Sabouraud's Dextrose Agar (Appendix 1) and stored at 4^{o}C for up to 3 months or until used. For longer storage of isolates, conidia were resuspended in 20-30% glycerol suspensions and stored at -20^{o}C. These conidia were used as inoculum for fresh stock plates.

4.2.1.2. Preparation of Conidia.

Inoculated plates of each isolate were incubated at 28^{o}C for 10-14 days in the dark or until sporulation occurred. Conidia were harvested in 0.02% (w/v) Tween 80, washed and sonicated as described in section 2.2.2.

Spores were counted in an improved Neubauer haemocytometer and each sample was diluted to a final concentration of 1.5 x 10^{6} spores per ml.

Table 22: Isolates of *Metarhizium* spp. and their sources.

Isolate	Strain	Source	Host
330189 s.s.	*M. flavoviride*	Niger	Orthoptera:Acrididae
ARSEF 324	*M. flavoviride*	Australia	Orthoptera:Acrididae
324673	*M. flavoviride*	Tanzania	Orthoptera:Pyrgomorphidae
ARSEF 2023	*M. flavoviride*	Galapagos Is.	Orthoptera:Acrididae
ARSEF 438	*M. anisopliae*	Australia	Orthoptera:Gryllidae
ARSEF 439	*M. anisopliae*	Australia	Orthoptera:Gryllidae
ARSEF 440	*M. anisopliae*	Australia	Orthoptera:Gryllidae
ARSEF 727ii	*M. anisopliae*	Brazil	Orthoptera:Tettigonidae
I90574	*M. anisopliae*	Pakistan	Orthoptera:Acrididae
I91633	*M. anisopliae*	Oman	Orthoptera:Gryllidae
I91676	*M. anisopliae*	Pakistan	Orthoptera:Acrididae
298059	*M. anisopliae*	PNG	Coleoptera:Scarabaeoidae
298061	*M. anisopliae*	PNG	Coleoptera:Hispidae
299981	*M. anisopliae*	Trinidad	Hemiptera:Cercopidae
299981	*M. anisopliae*	Trinidad	Hemiptera:Cercopidae
ME1	*M. anisopliae*	USA	Coleoptera:Curculionidae
Nr48	*M. anisopliae*	Thailand	Orthoptera:Acrididae
152222	*M. anisopliae*	India	Coleoptera:Curculionidae
168777ii	*M. anisopliae*	Ethiopia	Orthoptera:Acrididae

4.2.1.3. Preparation of fungal cultures.

Shake cultures of all isolates were used to study the production of cuticle degrading proteinases (CDPs) *in vitro*. For time course studies of CDP production, 50 ml aliquots of basal salts medium (Appendix 1) were dispensed into 100 ml Erlenmeyer flasks and supplemented with 1 % (w/v) ground whole locust body cuticle. After sterilisation by autoclaving at 121^{o}C for 15 minutes, flasks were inoculated with 1.5 x 10^{5} conidiospores of a particular isolate. The flasks were incubated at 140 rpm and 28^{o}C for up to 7 days in the dark and , a 200 µl aliquot of each culture was removed every 24h for analysis. Aliquots were centrifuged at 13000 rpm for 4 min in a microcentrifuge and the supernatant removed and stored at -20°C until used. Duplicate cultures for each strain were prepared and analysed.

4.2.2. Preparation of cuticle.

4.2.2.1. Preparation of cuticle from *S. gregaria*.

A pure preparation of adult locust cuticle was prepared by the method of Andersen (1980) and Andersen and Roepstrorff (1978). Approximately 100 locusts were killed by freezing at -20°C and homogenised in a Waring blender in 1 litre of 1 % (w/v) potassium tetraborate (K Borate). The suspension was sieved (1 mm pore diameter) and the retained material re-homogenised and washed several times with K Borate. The remaining insoluble material was then stirred overnight at 4°C in 2 litre of 1 % K Borate. It was then filtered, washed several times with distilled water and air dried. The dried cuticle was then milled to a fine powder in a Glen Creston-Ball Mill (DEH 48) using a 0.2 mm sieve. The resulting powder was kept at room temperature until used.

This method was used also to prepare wing cuticle and abdominal cuticle. Frozen locusts were thawed and the appropriate body parts removed and processed as described above..

To prepare pharate adult abdominal cuticle, 5th instar nymphs of *S. gregaria* were taken immediately prior to ecdysis. This stage was indicated by a colour change from yellow to light pink on the legs and lower abdomen of the nymph. The insects were killed by decapitation. The thorax was detached from the abdomen. Then the remains of the 5th instar cuticle were peeled away from the abdomen with a pair of fine forceps to reveal new unsclerotised adult cuticle underneath. Approximately 60 nymphs were sacrificed to yield 150 mg cuticle. Preparation of the ground cuticle was performed as above.

4.2.2.2. Preparation of [^{3}H]-labelled cuticle from *M. sexta*.

Twelve *Manduca* pupae were injected with 25 µCi of L-[4,5-^{3}H]leucine (60 Ci per mMol., Amersham, U.K.) 10 days prior to adult ecdysis and pupal cuticles removed 5 days after injection. Ground cuticle was prepared by the method of Andersen (1980) as described above. However, after washing with K Borate overnight, the cuticle was washed 6 times with distilled water to remove any unincorporated radiolabel.

4.2.3. Isolation of Proteolytic enzymes.

In order to develop an enzyme linked immunoadsorbant assay (ELISA) to PR1, pure PR1 was required for immunisation and subsequent testing in assays.

4.2.3.1. Isolation of PR1 and PR2 from *M. anisopliae* (strain ME1).

A 700 ml shake culture of basal salts medium supplemented with 1% (w/v) ground whole body cuticle was prepared. After sterilisation, the culture was inoculated with 1.5 x 10^5 spores and incubated as before for 7 days. The culture was then filtered in a Buchner funnel through Whatman No 1 filter paper. The filtrate was separated into 3 aliquots and frozen in round-bottomed flasks. Filtrates were then freeze-dried over 2 days until approximately 15 ml remained in each flask. The sample (approx. total volume 50 ml) was filtered through a 0.45 μm filter (Gelman, New Jersey), dialysed against distilled water overnight and stored at -20^{o}C until used.

4.2.3.2. Anion exchange chromatography.

To remove PR2, concentrated culture filtrates were passed down a sepharose Q ion exchange column in batches. A Q-sepharose column (20 cm x 1 cm), equilibrated in Tris-HCl buffer (10 mM; pH 7.0) was loaded with 10 ml of sample and unbound material eluted with 5 column volumes of the same buffer at a flow rate of 0.8 ml/min. The eluate (which contained PR1 activity) was collected in a round-bottomed flask and frozen. After loading, the PR2 activity was eluted with a salt gradient (Buffer A: 10 mM Tris-HCl; pH 7.O: Buffer B: 10 mM Tris-HCl with 2 M NaCl; pH 7.0). Fractions were collected using an automated fraction collector (Pharmacia) and the absorbance at 280 nm of each fraction determined. Those fractions containing PR2 activity were dialysed against water and passed down the Q-sepharose column again under the same conditions except that buffer B in the gradient was 10 mM Tris-HCl with 1 M NaCl (pH 7.0). Fractions containing PR2 activity were dialysed against water overnight and freeze-dried to a final volume of 1 ml.

4.2.3.3. Cation exchange high performance liquid chromatography.

The eluate from anion exchange chromatography was freeze dried to a powder and resuspended in 5 ml distilled water. After overnight dialysis against distilled water and subsequent freeze-drying, the enzyme activity was recovered in 2 ml MilliQ water.

This sample was then fractionated on an ion exchange column under high pressure run on a Gilson system consisting of two model 303 pumps, an 811B dynamic mixer, a 401 dilutor, a model 231 sample injector and Rheodyne injection valve fitted with a 20 µl, 100 µl or 500µl sample loop. The chromatography unit was controlled by an Opus computer which recorded the output from a 116 UV detector set at a wavelength of 280nm.

High performance ion exchange chromatography used a Hydropore^{TM}-SCX cation exchange column (Rainin Instrument Co.) with a guard column of the same material. The column was equilibrated with 0.02 M sodium acetate buffer (pH 6.0) made up in Milli-Q water. The sample was loaded in Milli-Q water and eluted with a linear gradient of 0.02 M sodium acetate buffer containing 0.4 M NaCl (pH 6.0; 0-25% gradient) at a constant flow rate of 1.00 ml per minute. Both mobile phases were degassed with nitrogen prior to use. Fractions were collected as peaks became visible (on the monitor) using a programmable Gilson 201-202 fraction collector.

All fractions were assayed for PR1 activity and those containing enzyme were dialysed overnight against water and subsequently freeze-dried. After lyophilisation, the fractions were resuspended in 1000 µl Milli-Q water and stored at -20°C until required. Purity of the enzyme preparation was determined by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE).

4.2.3.4. Partial Purification of PR1 from 19 strains of *Metarhizium* sp.

In order to assess the ability of PR1 from each strain of *Metarhizium* to degrade cuticle, it was necessary to partially purify the enzyme from cultures of each isolate. Shake cultures (250 ml) of each strain were grown on ground adult *S. gregaria* whole body cuticle, filtered and processed as described above (section 4.2.3.1.). The treated culture filtrates were then passed down a Q-column as before and the eluent collected and freeze-dried to a powder. After resuspending and dialysing against distilled water, each sample was loaded onto a S-Sepharose column.

A S-Sepharose column (20 cm x 1 cm), equilibrated in Sodium acetate buffer (20 mM; pH 6.0) was loaded with 15 ml of culture filtrate and unbound material was eluted immediately by washing with 5 column volumes of the same buffer at a flow rate of 0.8 ml/min. The eluate (which contained PR1 activity) was collected in a round-bottomed flask and frozen. After loading, the PR2 activity was eluted with a salt gradient (Buffer A: 20 mM sodium acetate; pH 6.0: Buffer B: 20 mM sodium acetate and 400 mM NaCl; pH 7.0). Fractions were collected using an automated fraction collector (Pharmacia) and the absorbance at 280 nm of each fraction taken. Those fractions containing PR1 activity were dialysed against water and freeze-dried to a final volume of 1 ml. These samples were assayed for PR1 activity both with and without preincubation with the enzyme inhibitor, Turkey egg white inhibitor (TEWI) in order to confirm the presence of PR1. Furthermore the samples were assayed against Benzoyl-Phe-Val-Arg-pNa which is a substrate for PR2 and PR4 activity. If mono-endoprotease activity was detected, the samples were used for cuticle digestion studies and ELISA. Table 23 shows the amount

Table 23. PR1 activity after S-sepharose chromatography of concentrated culture filtrates of 19 strains of *Metarhizium* Spp.

Strain [a]	PR1 activity [b] (Units/ml)
3330189[a]	170
ARSEF 324[a]	390
324673[a]	1290
ARSEF 2023[a]	470
ARSEF 438	520
ARSEF 439	2400
ARSEF 440	210
ARSEF 727ii	80
I90574	76
I91633	240
I91676	670
298059	170
298061	120
299981	58
299984	280
ME1	200
Nr48	250
152222	334
168777ii	200

[a] Indicates *M. flavoviride* isolate, the rest are *M. anisopliae.*

[b] Enzyme activity determined against synthetic nitroanilide substrates (see materials and methods). One unit of enzyme activity was defined as that amount of enzyme that increased the absorbance reading at a rate of 0.001 absorbance units per minute

of activity recovered for each isolate. These values were used to calculate the amount of material released from cuticle per unit PR1 activity.

4.2.4. Cuticle digestion.

For cuticle digestion assays on *Manduca* pupal cuticle, aliquots of labelled cuticle were pre-washed in 500 µl 10 mM Tris-HCl, 5mM $CaCl_2$, pH 8.0 for 1h. This buffer was removed and fresh buffer added prior to addition of samples. The final assay contained 3.3 ± 0.07 mg of ground cuticle suspended in 50 µl PR1 in milliQ water and 950 µl 10 mM Tris-HCl buffer (pH 8.0). Incubations performed were a) PR1 alone, b) buffer alone, c) PR1 preincubated for 20 min on ice with turkey egg white inhibitor (1mg/ml) and d) PR1 without cuticle. The samples were incubated at 28°C on a cyclogyrator for 6h. Following this they were centrifuged (13000 x rpm; 3 min, room temperature) and the supernatant removed. One hundred µl aliquots of sample were then added to 5 ml liquid scintillant (Optiphase Safe; LKB) and the released ^{3}H-peptides counted using a liquid scintillation spectrophotometer in a LKB Rackbeta counter. Quenching was automatically corrected for by use of an external standard.

For cuticle digestion assays on locust cuticle a modification of the method described by St. Leger *et al.* (1991) was used, the samples were washed in 1 ml 2 mM Tris-HCl, pH 8.0 for 1 h. The buffer was removed and fresh buffer added prior to the addition of samples. The amount of cuticle in each sample was 5.3 ± 0.13 mg (abdominal cuticle), 5.2 ± 0.13 mg (wing cuticle) and 1.5 ± 0.05 (pharate cuticle). For each cuticle type, the final assay contained cuticle suspended in 50 µl PR1 in milliQ and 950 µl 10 mM Tris-HCl buffer (pH 8.0). Incubations performed were a) PR1 alone, b) buffer alone, c) PR1 preincubated for 20 min on ice with turkey

egg white inhibitor (1mg/ml) and d) PR1 without cuticle. The samples were incubated at 28°C on a cyclogyrator for 6h. Following this they were centrifuged (13000 x rpm; 3 min, room temperature) and the supernatant removed. Samples were stored at -20°C until used.

4.2.5. Protein determination.

For purification steps, total protein was determined by the Bradford Method (Bradford, 1976) using a protein assay kit (Bio-Rad, U.K.). A standard curve was prepared each time using a stock solution of bovine gamma globulin. The absorbance of samples was measured at 595 nm.

For cuticle digestion samples (section 4.2.4), the absorbance at 260 and 280 nm was taken using a Cecil 2000 series spectrophotometer (Cecil Instruments, Cambridge, U.K.). Protein concentration in mg per ml was determined using the expression:

$$(1.55 \times A_{280}) - (0.76 \times A_{260})$$

The value obtained from this expression, P, was then put in the following expression:

$$P/(\text{mg cuticle} \times \text{Units PR1 activity})$$

This gives the amount of protein released per mg cuticle per unit PR1 activity.

4.2.6. Ninhydrin assay.

To determine the amount of soluble nitrogen liberated by PR1 protease action (section 4.2.4.), 100 µl aliquots of samples were added to

ninhydrin reagent. This was prepared immediately prior to use by mixing equal volumes of 4 % (w/v) ninhydrin in 2-methoxyethanol with 0.2 M citrate buffer containing 0.2 % (w/v) $SnCl_2$ (pH 5.0). The reaction mixture was then boiled for 20 min in capped test tubes. After cooling, 3 ml 50 % (v/v) propan-2-ol was added prior to measuring the absorbance at 570 nm. A calibration curve was generated for each assay using alanine as a standard. Results were expressed in µg Alanine equivalents released per ml. The values obtained, N, were then put in the expression:

N/(mg cuticle x Units PR1 activity)

This gives the amount of alanine equivalents released per mg cuticle per unit PR1 activity.

4.2.7. Enzyme Assays.

4.2.7.1. PR1 assay.

PR1 activity was determined using the chromogenic oligopeptide substrate Succinyl-Ala-Ala-Pro-Phe-pNA (Sigma; Del Mar *et al.*,1979). To 50 µl of enzyme sample was added 900 µl 0.1M Tris-HCl buffer (pH 8.0). The reaction was initiated by the addition of 50 µl 1mM substrate. The change in absorbance at 410nm over 1 min was recorded using a Pye Unicam PU8650 visible spectrophotometer (Philips). One unit of enzyme activity was defined as that amount of enzyme that increased the absorbance reading at a rate of 0.001 absorbance units per minute.

4.2.7.2. PR2 assay.

PR2 activity was determined using the chromogenic oligopeptide substrate Benzoyl-Phe-Val-Arg-pNA (Sigma). To 50 µl of enzyme sample was added 900 µl 0.1M Tris-HCl buffer (pH 8.0). The reaction was initiated by the addition of 50 µl 1mM substrate. The change in absorbance at 410nm over 1 min was recorded using a Pye Unicam PU8650 visible spectrophotometer (Philips). One unit of enzyme activity was defined as that amount of enzyme that increased the absorbance reading at a rate of 0.001 absorbance units per minute.

4.2.7.3. Fluorimetric assay for PR1.

PR1 activity was also determined using the fluorogenic oligopeptide substrate Succinyl-Ala-Ala-Pro-Phe-4-methyl-coumaryl-7-amide (Calbio-chem; Irvine *et al.*, 1990) dissolved in DMSO. Using a 96-well microtiter fluorography plate, 50 µl of enzyme sample was added to 100 µl 0.1M Tris-HCl buffer (pH 8.0). The reaction was initiated by the addition of 50 µl 1mM substrate. The change in fluorescence over 10 min was recorded using a Fluoroskan II (Titertek) measuring at an emission wavelength of 460nm and an excitation wavelength of 355nm. Enzyme activity was determined by a deflection in the fluorescence when compared to control wells containing fluor and buffer alone. One unit of enzyme activity was defined as that amount of enzyme that increased the absorbance and emission reading at a rate of 0.001 units per minute.

4.2.9. Development of an Enzyme-linked Immunoadsorbent assay (ELISA) to detect PR1.

4.2.9.1. Raising antibodies to PR1

The method employed was essentially that described by St. Leger *et al.* (1987a). Briefly, 2 g of cyanogen bromide-activated sepharose was swollen. Once swollen, 50 mg chicken egg white ovoinhibitor (Sigma) was dissolved in coupling buffer (0.1M $NaHCO_3$; 0.5M NaCl; pH 8.3) and added to the gel and agitated end-over-end for 16 h at 4°C. The gel was then washed with 10 vols coupling buffer, suspended in 20 ml 0.1M Tris-HCl (pH 8.0) and incubated for 2 h at room temperature to block unreacted groups. The sepharose was washed with 5 vols coupling buffer, 0.1M sodium acetate, 0.5M NaCl (pH 4.0) and finally coupling buffer. the gel was suspended in 2 ml 50mM phosphate buffer containing 0.5M NaCl (pH 7.0) and adsorbed with 10 mg PR1 and stored at -20°C in 10 x 1 ml aliquots. These samples were sent to Shell Research Station, Sittingbourne, Kent where they were injected into a rabbit by Dr. P. Aston. One rabbit was injected intradermally with a 1:1 dilution of antigen in complete Freunds adjuvant. A secondary boost of antigen in incomplete Freunds adjuvant was injected subcutaneously. The rabbits were bled from an ear vein 10 d after the secondary boost. A tertiary boost of pure PR1 emulsified in Freunds complete adjuvant was prepared and injected into the rabbit subcutaneously. This was done to clonally expand the polyclonal antibodies raised against PR1 and not ovoinhibitor or the PR1-ovoinhibitor bond(s). Twenty ml of blood was obtained from an ear bleed 10 days later.

Collected blood was allowed to stand on ice for 60 min. The serum was then centrifuged at 2400 rpm at 8°C for 20 min. the supernatant was decanted and re-centrifuged under the same conditions to remove all

particulate matter. IgG molecules were obtained by mixing the antiserum with 45% (v/v) saturated ammonium sulphate solution. The mixture was left to stand in ice for 20 min before being centrifuged at 8000 rpm and 4°C for 15 min. The resultant supernatant was then divided into 40 ul aliquots and frozen at -20°C until used in assay.

4.2.9.2. Enzyme-linked immunoadsorbant assay (ELISA).

Two types of ELISA were performed. These techniques allowed for the optimization of an assay as well as determining any cross-reactivity with other molecules.

4.2.9.2.1. Antigen-coated plate assay.

In order to quantify PR1 in an unknown sample, an ELISA assay was devised. Nunc immunoplates (Gibco) were coated with antigen (100 units PR1/well) in carbonate buffer (1.59g $NaCO_3$; 2.93g $NaHCO_3$; per l; pH 8.6) and left overnight at 4°C. The plates were then washed twice with PBS containing 0.05% (v/v) Tween 20 and coated with PBS containing 1% (w/v) gelatin to block any remaining sites on the plate that had not bound PR1. The plates were incubated for 1 h at room temperature and then washed twice with phosphate buffered saline (PBS) containing 0.05% (w/v) Tween 20. Antiserum was then added to the plates and diluted across the rows of the plate from 1/100 to 1/160000. The plate was incubated for 2 h at room temperature and washed 4 times with PBS-Tween. Goat anti-rabbit antiserum (Dako Pats) conjugated with horseradish peroxidase was then incubated in each well for 2 h at room temperature. The plate was then washed 5 times with PBS-Tween before addition of reagent. One ml ABTS (0.015g/ml) was diluted in 9 ml ABTS buffer (4.405g $NaHPO_4$;

4.575g citric acid; 180µl hydrogen peroxide; per 500 ml) and incubated for 30 min or until a visible colour change was observed. The plate was then read at 410nm in a plate reader (Dynatech) and the data analysed using the software package supplied with the machine.

Plates were prepared to test the detection limits of (a) different dilutions of the antiserum against a fixed concentration of antigen and (b) the detection limits of a fixed dilution of antiserum against different concentrations of antigen. In the latter case, antigen (PR1) was serially diluted from 10 µg/well to 0.04 µg/well. Antiserum at a working dilution of 1/400 was then used to detect the enzyme

4.2.9.2.1. Competitive Inhibition Assay.

This type of assay is designed to determine if the antibodies raised to the antigen (PR1) recognises binding sites on other molecules. This is not a quantitative assay and was used to identify the recognition properties of the antiserum.

The competitive inhibition assay was used to test against a) proteases from other sources b) PR1s isolated from other strains of *M. anisopliae* as well as *M. flavoviride* and c) uninfected and infected blood from *S. gregaria* and *M. sexta.*

The assays were performed as follows:

1) Proteases;

Stock solutions of 1 mg/ml of the following pure enzymes were prepared in PBS buffer: chymotrypsin, trypsin, proteinase K, subtilisin, thermolysin, papain, moulting fluid protease I (MFP-1; Samuels *et al.*, 1993) and elastase. A further enzyme, PR2, isolated partially pure from *M. anisopliae* (strain ME1) was also tested. A stock solution of 100 units/100

µl of each enzyme was used (One unit of enzyme activity was defined as that amount of enzyme that increased the absorbance reading at a rate of 0.001 absorbance units per minute against its respective substrate). One hundred µl of each enzyme solution was incubated with 100 µl of a 1/200 dilution of antiserum (final dilution factor of 1/400) for 1 h at room temperature. One hundred µl of each mixture was placed in a well in a microtiter plate coated with 100 units of PR1 and incubated for 2 h at room temperature. The plate was washed and developed as described in section 3.3.8.2.1.

2) PR1 proteases isolated from *Metarhizium* spp.

The same assay outlined above was performed to detect cross-reactivity with other PR1s except that, initially, 100 units of partially purified enzyme from each of the strains was pre-incubated with 100 µl of 1/200 dilution of antiserum. The assay was then performed as described above.

3) Insect blood.

In order to detect whether the antiserum cross-reacted with naive insect blood, a competitive inhibition assay was performed using blood from adult *S.gregaria* and *M. sexta* larvae.

Furthermore, in order to detect the presence of PR1 enzyme in the blood of infected insects, a competitive inhibition assay using the blood from infected insects was also performed.

4.2.10. Statistical analysis.

For the cuticle hydrolysis assays results were compared using Minitab (Minitab).

4.3. Results

4.3.1. The production of cuticle degrading proteases (PR1 and PR2) by *Metarhizium* spp.

All isolates of *Metarhizium* tested produced PR1 and PR2 in buffered minimal media cultures containing 1% (w/v) locust cuticle as the sole carbon and nitrogen source (Tables 24 and 25; Figs. 27-31). Low amounts of PR1 activity was observed as early as 48 h after inoculation in some cultures (Table 24). However, in the case of most of the strains tested, PR1 activity was observed after 72 h. The amounts of PR1 varied greatly between each isolate used. For example, the *M. flavoviride* isolate 330189 produced 7600 units per ml culture fluid after 6 days whilst the *M. anisopliae* strain 168777ii produced 500 units per ml after the same amount of time. As can be seen by figures 1-5, there is a large increase in PR1 activity in almost all of the isolates between 4 and 6 days. PR2 was detected in most strains as early as 48 h after inoculation (Table 25).

There was no significant correlation between median lethal time for each strain (Table 26; data supplied by Dr. C. Prior, IIBC, Silwood Park, Ascot: see appendix 3) and PR1 activity at day 6 ($P>0.1$).

4.3.1.2. Purification of PR1 and PR2.

The method Patterson (1992) used to isolate PR1 was tried and appeared to be wasteful in the sense that only a small amount of PR1 was recovered from a large amount of culture fluid. I modified the method by using batch purification and, in doing so, was able to retain PR2 for experimental purposes. Figure 32 shows the protocol employed to achieve this.

Table 24. The production of PR1 activity by 19 strains of 2 species of *Metarhizium* spp. during 72 hours after inoculation on 1% ground adult locust whole body cuticle.

Strain	PR1 activity[a]		
	24 h	48h	72h
330189[b]	0	0	45
324673[b]	0	0	64
ARSEF 324[b]	0	0	51
ARSEF 2023[b]	0	0	93
ARSEF 438	0	0	148
ARSEF 439	0	0	188
ARSEF 440	0	0	43
ARSEF 727ii	0	0	120
I90574	0	0	107
I91633	0	0	1
I91676	0	0	68
298059	0	10	50
298061	0	52	320
2999810	0	0	10
299984	0	0	0
ME1	0	0	72
Nr48	0	0	16
152222	0	0	25
168777ii	0	0	24

[a] Activity determined against Succ-Ala-Ala-Pro-Phe-pNA.Activity is recorded in Units. One unit of PR1 activity represents a change in absorbance of 0.001 per min at 410 nm.
[b] Indicates *M. flavoviride* species, the rest are *M. anisopliae*

Table 25. The production of PR2 activity by 19 strains of 2 species of *Metarhizium* spp. during 72 hours after inoculation after inoculation on 1% ground adult locust whole body cuticle.

Strain	PR2 activity[a]		
	24 h	48h	72h
330189[b]	0	10	50
324673[b]	0	8	19
ARSEF 324[b]	0	12	36
ARSEF 2023[b]	0	19	63
ARSEF 438	0	24	56
ARSEF 439	0	52	162
ARSEF 440	0	39	183
ARSEF 727ii	0	21	339
I90574	0	53	162
I91633	0	0	3
I91676	0	71	190
298059	0	15	56
298061	0	33	836
2999810	0	8	30
299984	0	0	10
ME1	0	21	72
Nr48	0	13	29
152222	0	25	57
168777ii	0	39	86

[a] Activity determined against Bz-Phe-Val-Arg-pNA. Activity is recorded in Units. One unit of PR2 activity represents a change in absorbance of 0.001 per min at 410 nm.
[b] Indicates *M. flavoviride* species, the rest are *M. anisopliae*

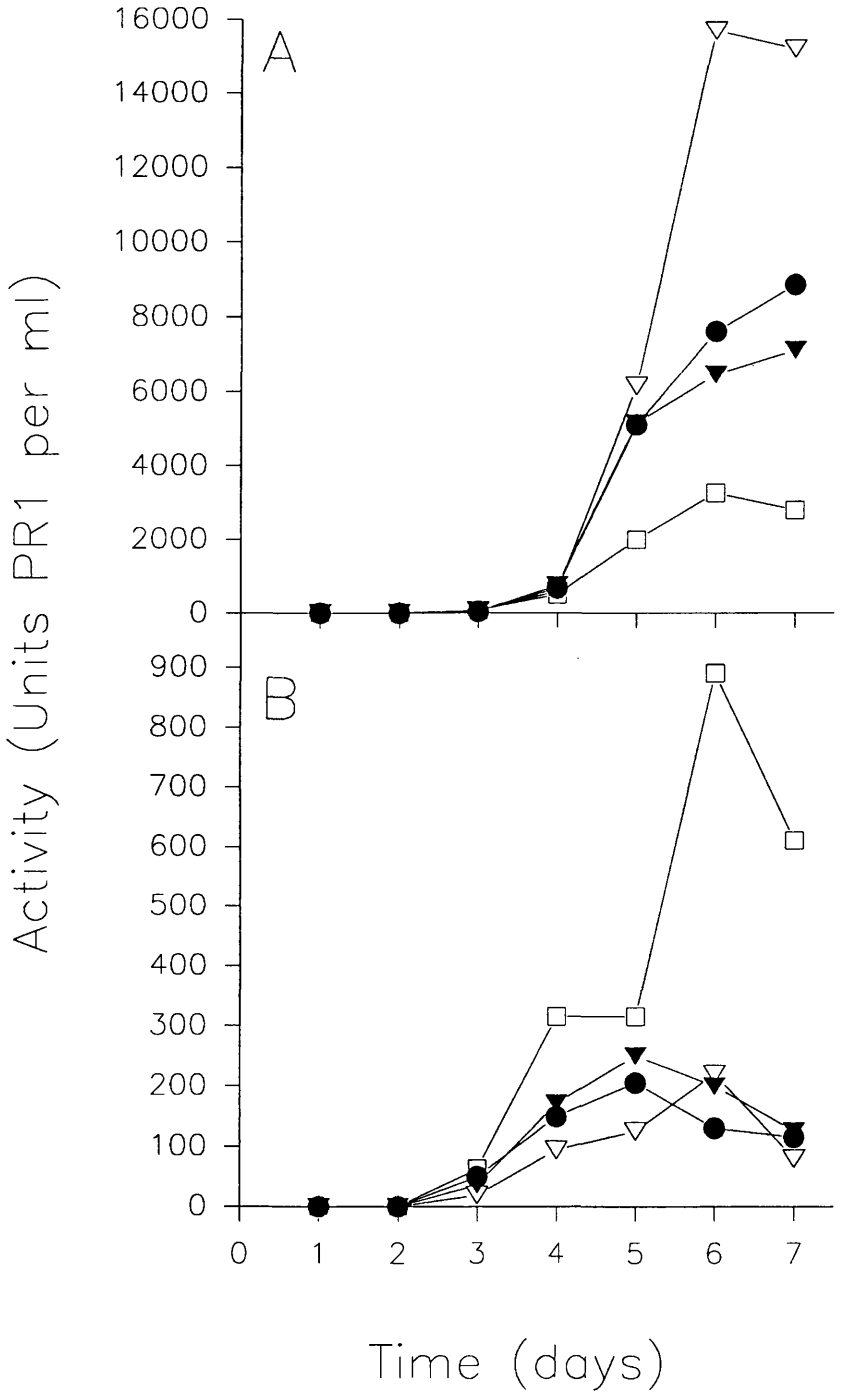


Figure 27. The production of (A) PR1 and (B) PR2 by isolates of *M. flavoviride* grown on 1% (w/v) cuticle.
● 330189 (S.S); ▽ 324673; ▼ ARSEF 324;
□ ARSEF 2023

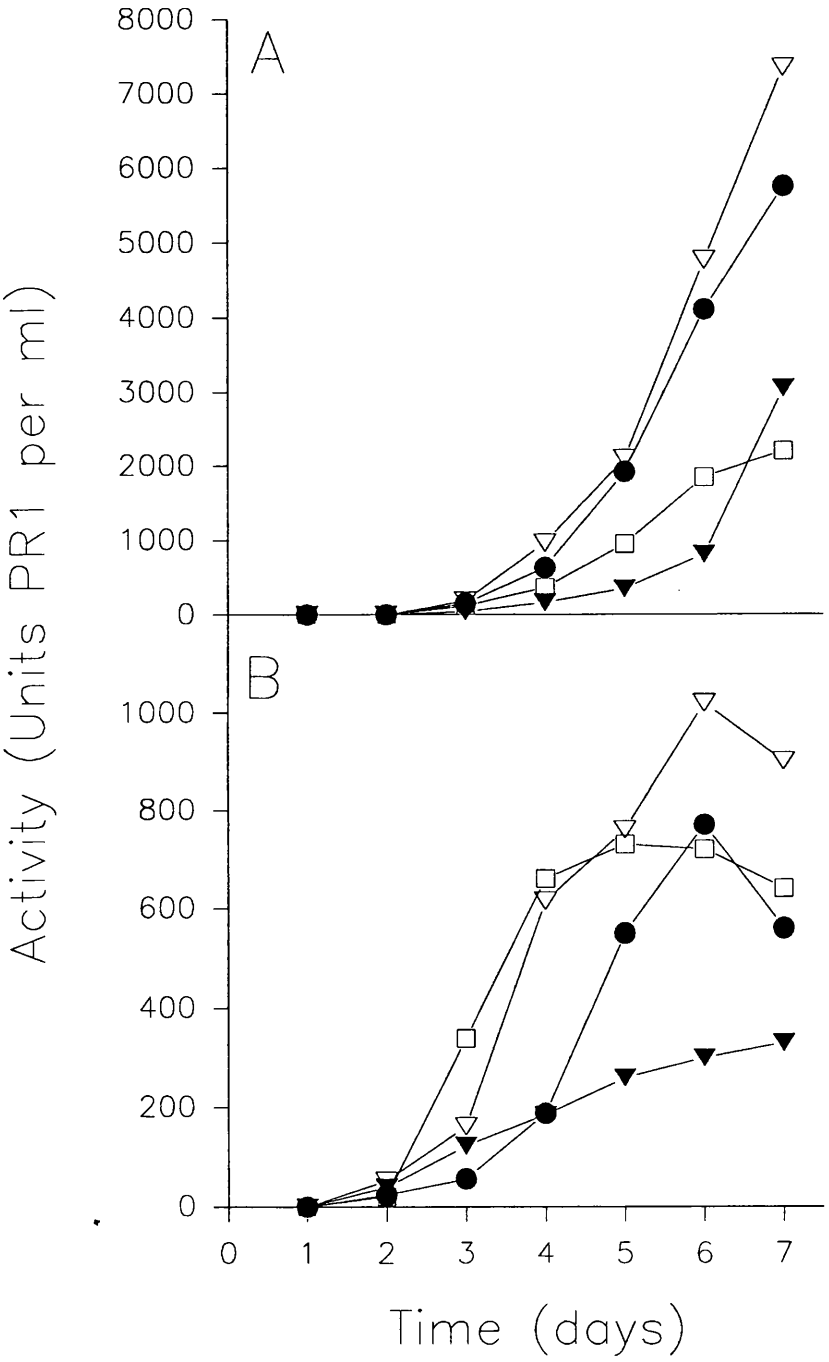


Figure 28. The production of (A) PR1 and (B) PR2 by isolates of *M. anisopliae* grown on 1% (w/v) cuticle. ● ARSEF 438; ▽ ARSEF 439; ▼ ARSEF 440; □ ARSEF 727ii.

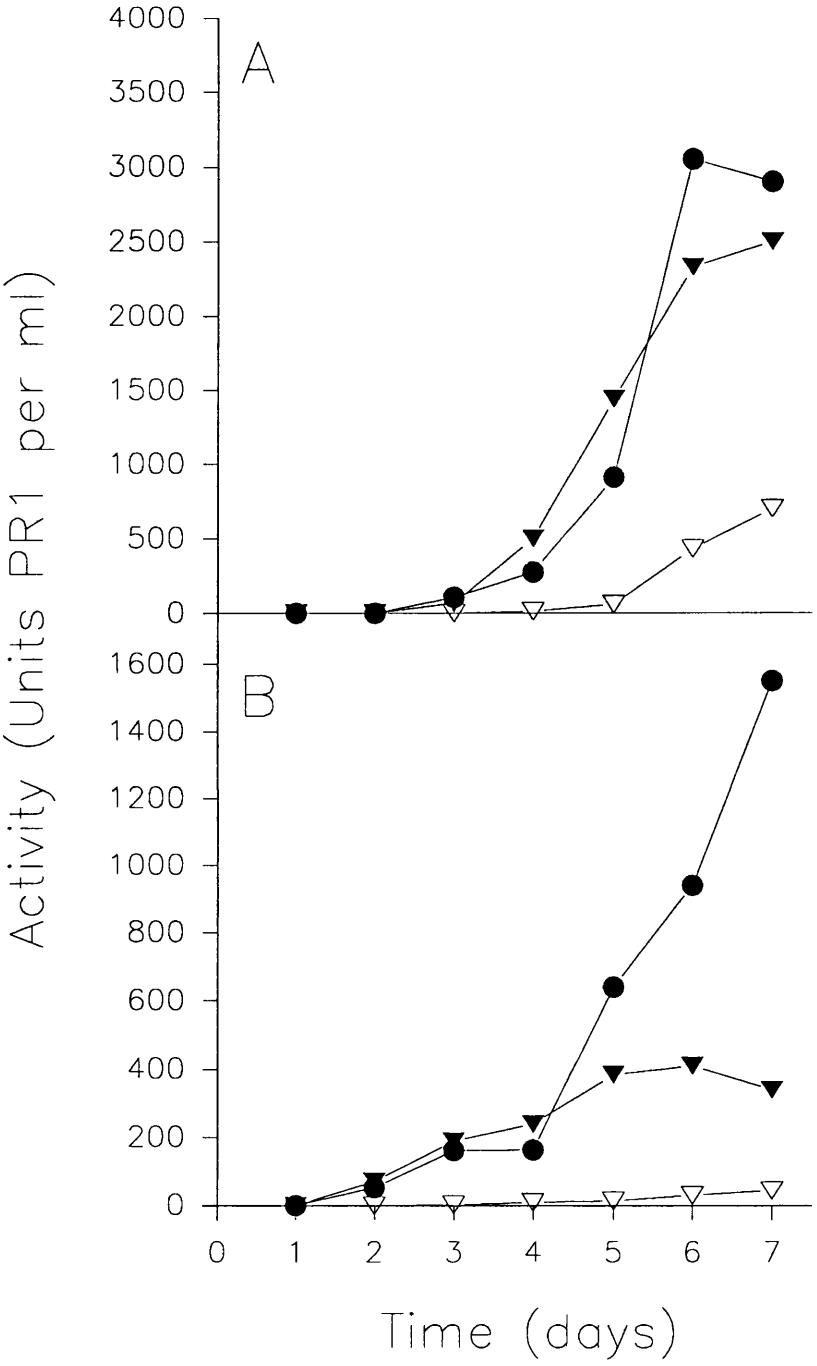


Figure 29. The production of (A) PR1 and (B) PR2 by isolates of *M. anisopliae* grown on 1% (w/v) cuticle. ● I90574; ▽ I91633; ▼ I91676.

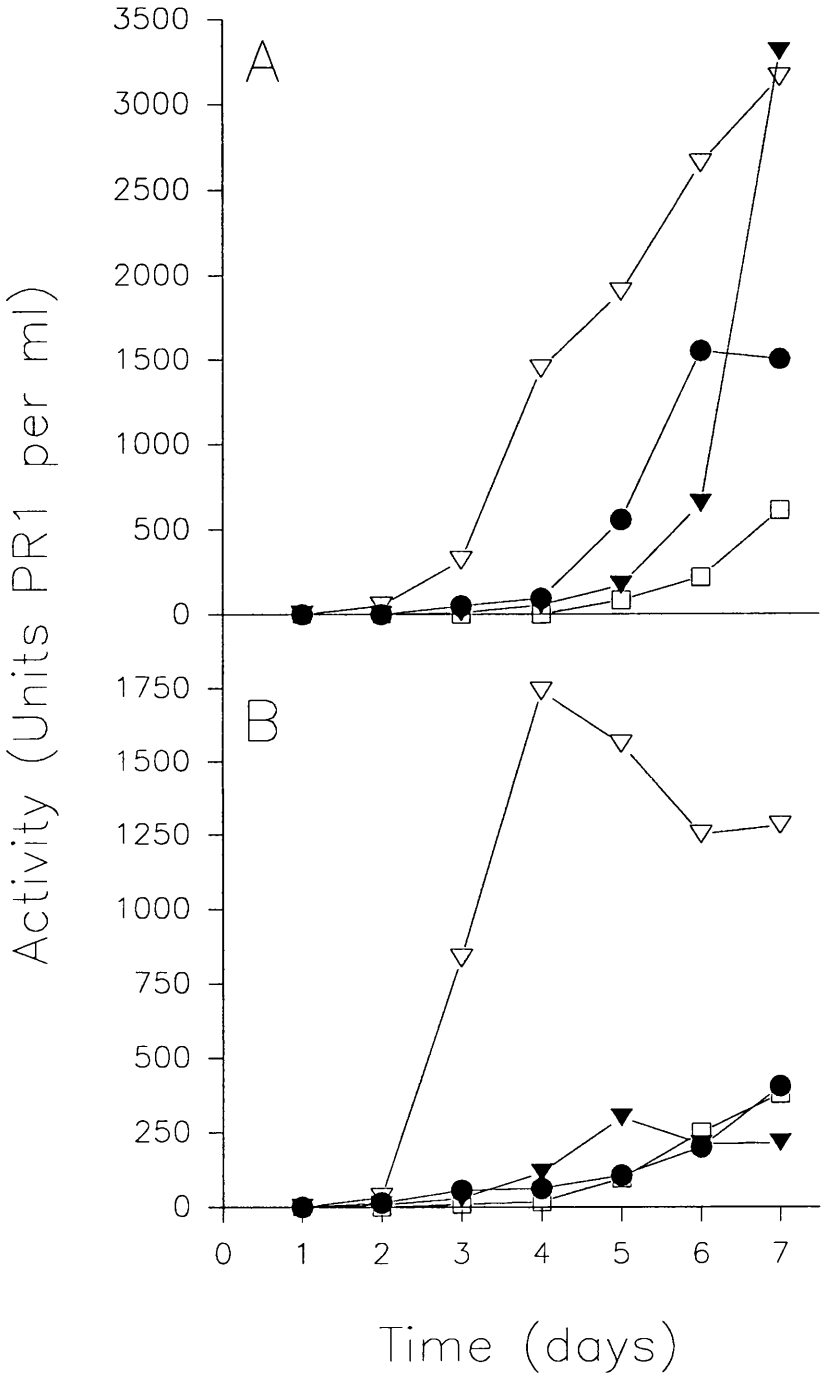


Figure 30. The production of (A) PR1 and (B) PR2 by isolates of *M. anisopliae* grown on 1% (w/v) cuticle.
● 298059; ▽ 298061; ▼ 299981; □ 299984

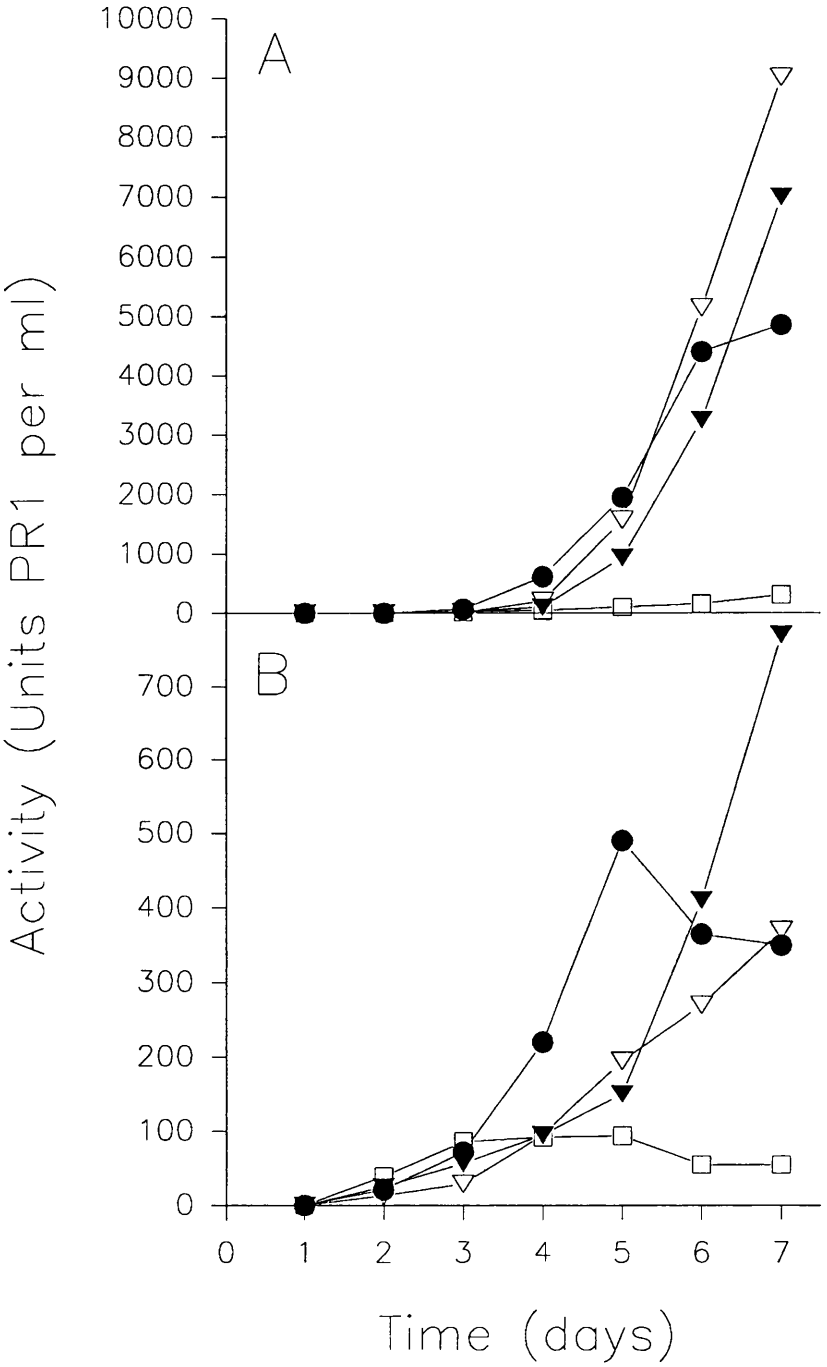


Figure 31. The production of (A) PR1 and (B) PR2 by isolatess of *M. anisopliae* grown on 1% (w/v) cuticle.
● ME1; ▽ Nr48; ▼ 152222; □ 168777ii.

Table 26. The median lethal time (MLT) for each isolate of *Metarhizium* spp. used.

Strain	MLT[b]
3330189[a]	4.41
ARSEF 324[a]	3.71
324673[a]	4.59
ARSEF 2023[a]	5.39
ARSEF 438	13.00[c]
ARSEF 439	13.00[c]
ARSEF 440	13.00[c]
ARSEF 727ii	10.50
I90574	11.12
I91633	13.00[c]
I91676	6.05
298059	8.02
298061	7.42
299981	5.93
299984	5.06
ME1	4.82
Nr48	4.83
152222	5.48
168777ii	4.96

[a] Indicates *M. flavoviride* species.
[b] Data supplied by Dr. C. Prior, IIBC, Silwood Park, Ascot.
[c] The median lethal time was not determined for these strains as less than 50% of experimental insects had died by the end of the observation period. These strains were given a value of 13, although the true value may have been greater.

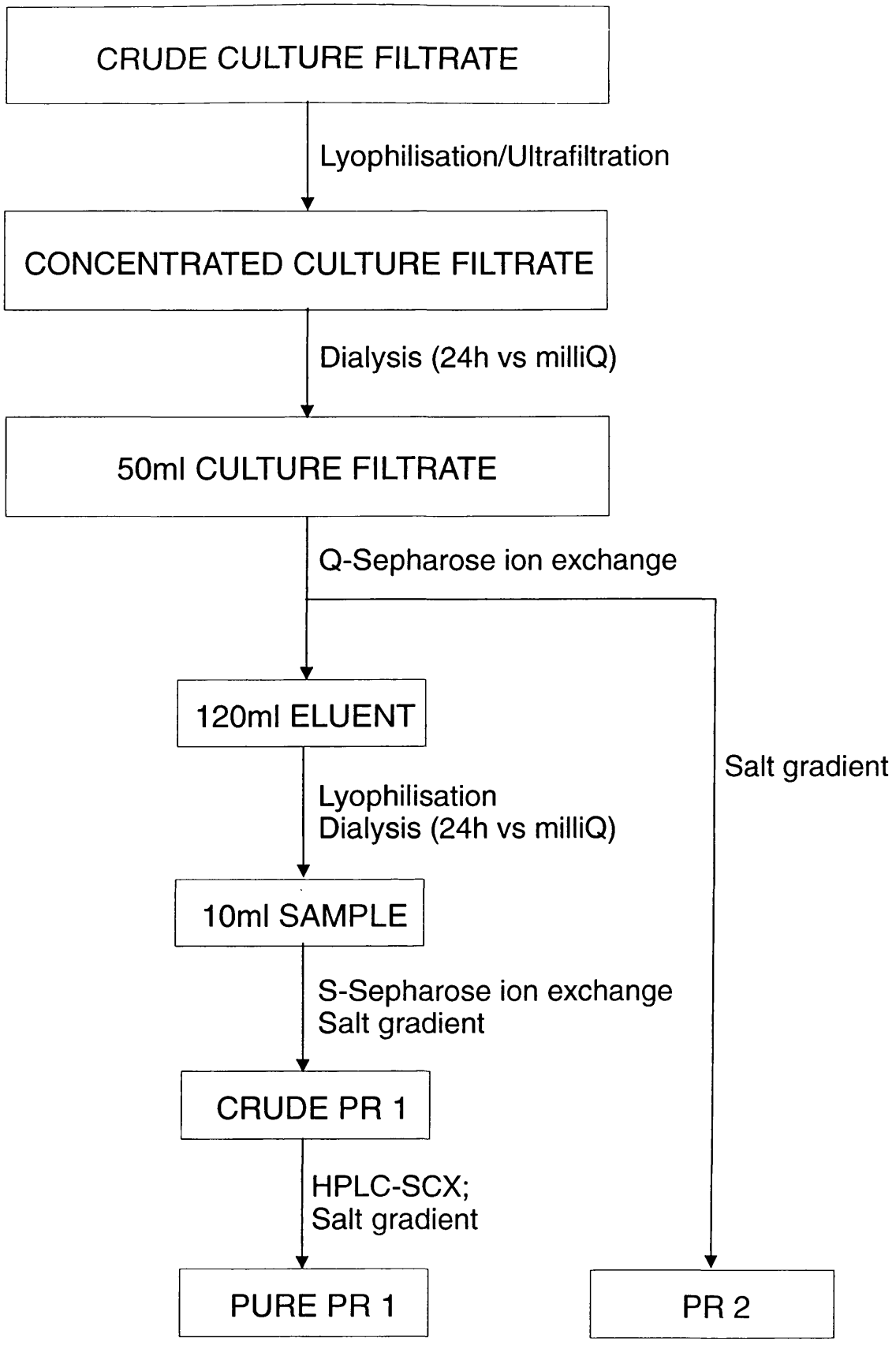


Figure 32. Protocol to show simultaneous extraction of PR1 and PR2 from culture filtrates of *Metarhizium* spp.(See Materials and Methods for details)

Ten ml aliquots of concentrated culture filtrates were applied onto a Q-sepharose column. PR1 was eluted with 5 column volumes of mobile phase. PR2 was eluted with a salt gradient. The enzyme appeared as a single broad peak at low salt concentration (figure 33). Attempts to process large volumes of filtrate (50 ml) resulted in overloading of the column as indicated by the appearance of a brown pigment (normally retained on the column) in the eluent.

The eluents from the batch Q-column runs were pooled and freeze-dried to a final volume of 5 ml before being applied to a S-sepharose (cation exchange) column. The PR1 was eluted with a NaCl gradient and enzyme activity identified (Figure 34). The peaks showing PR1 activity were pooled, dialysed and freeze-dried to a volume of 1 ml before passage on an HPLC-cation exchange system. The enzyme was eluted with 0.4 M NaCl. The arrowed peak in Figure 35 identifies PR1 and represents 37 % of the protein loaded onto the column. The initial peak consisted of material that didn't bind to the column and contained no PR1 activity. The other peaks seen during the elution are probably autodegradation products of PR1 (Patterson, 1992). The final yield of PR1 from the HPLC cation exchange column was 37% of the initial protein load cf. only 8% using the original method (Patterson, 1992; see figures 35 and 36).

4.3.1.3 Partial purification of PR1 enzymes from different strains of *Metarhizium* Spp.

The purification to homogeneity of PR1 from *M. anisopliae* (strain ME1) was necessary for the development of a high affinity antiserum to the enzyme (see later). In the case of the other strains of the fungus it was not important to purify to homogeneity. The purpose of these enzymes was to measure the hydrolysis of insect cuticle. For this, it was important that

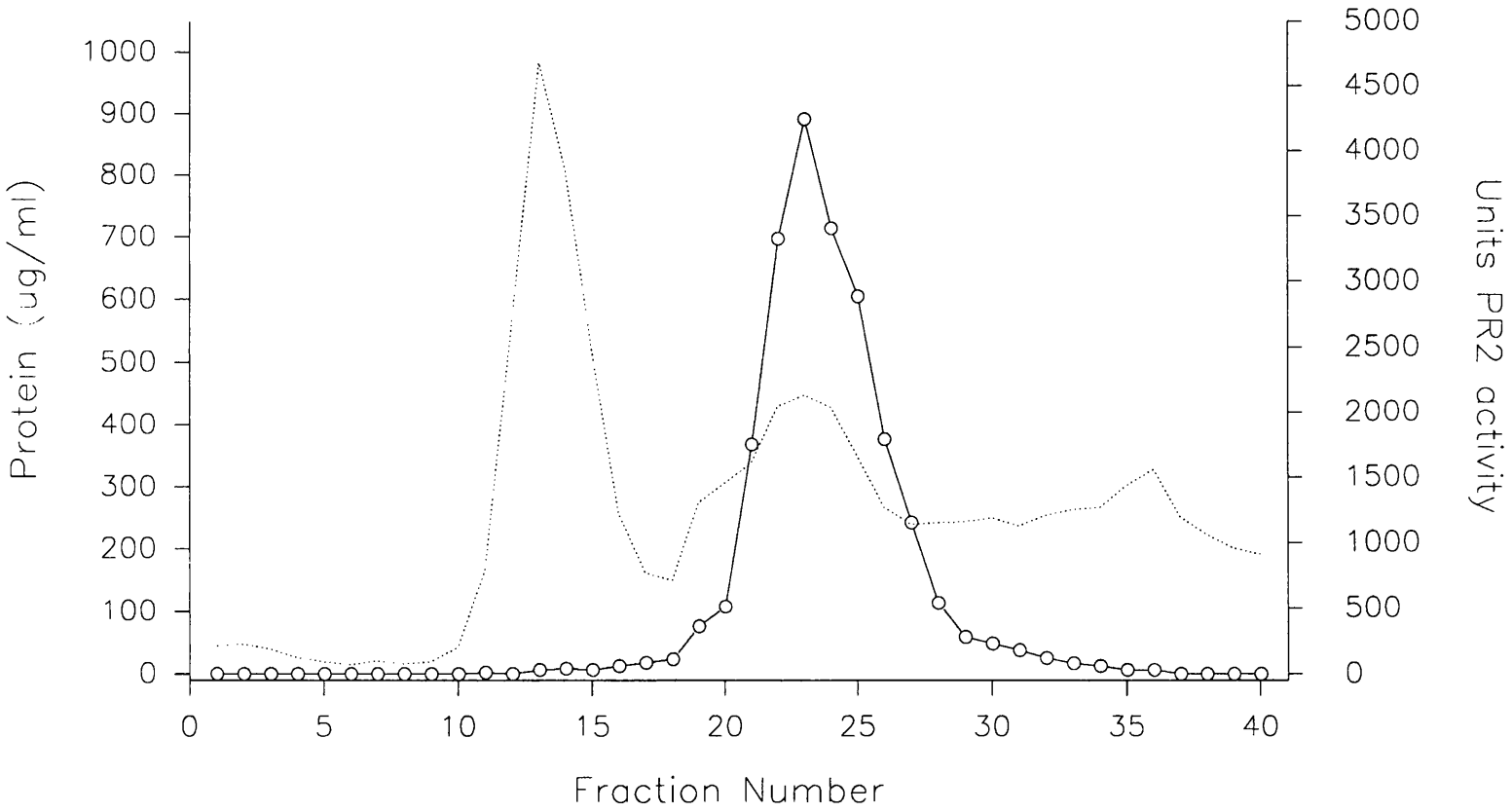


Figure 33. Elution profile of PR2 activity from *M. anisopliae* (isolate ME1) on Q–sepharose. The activity was recovered with a 1.0M NaCl gradient. (····) Protein concentration; (o) PR2 activity.

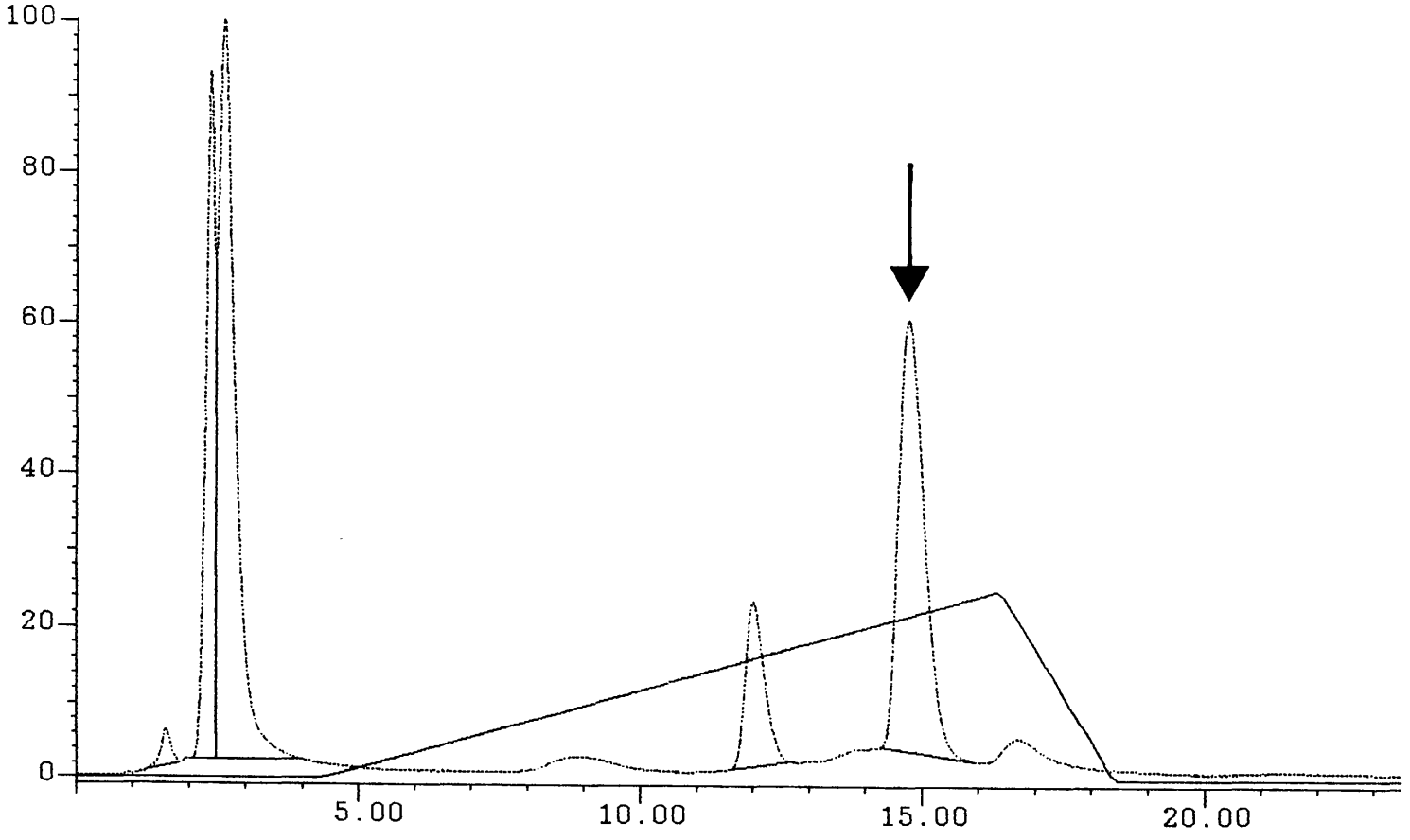


Figure 35. Chromatogram of PR1 from a crude culture filtrate of *M. anisopliae* (isolate ME1) fractionated on a strong cation exchange HPLC column. PR1 (arrowed) was eluted with a NaCl gradient.

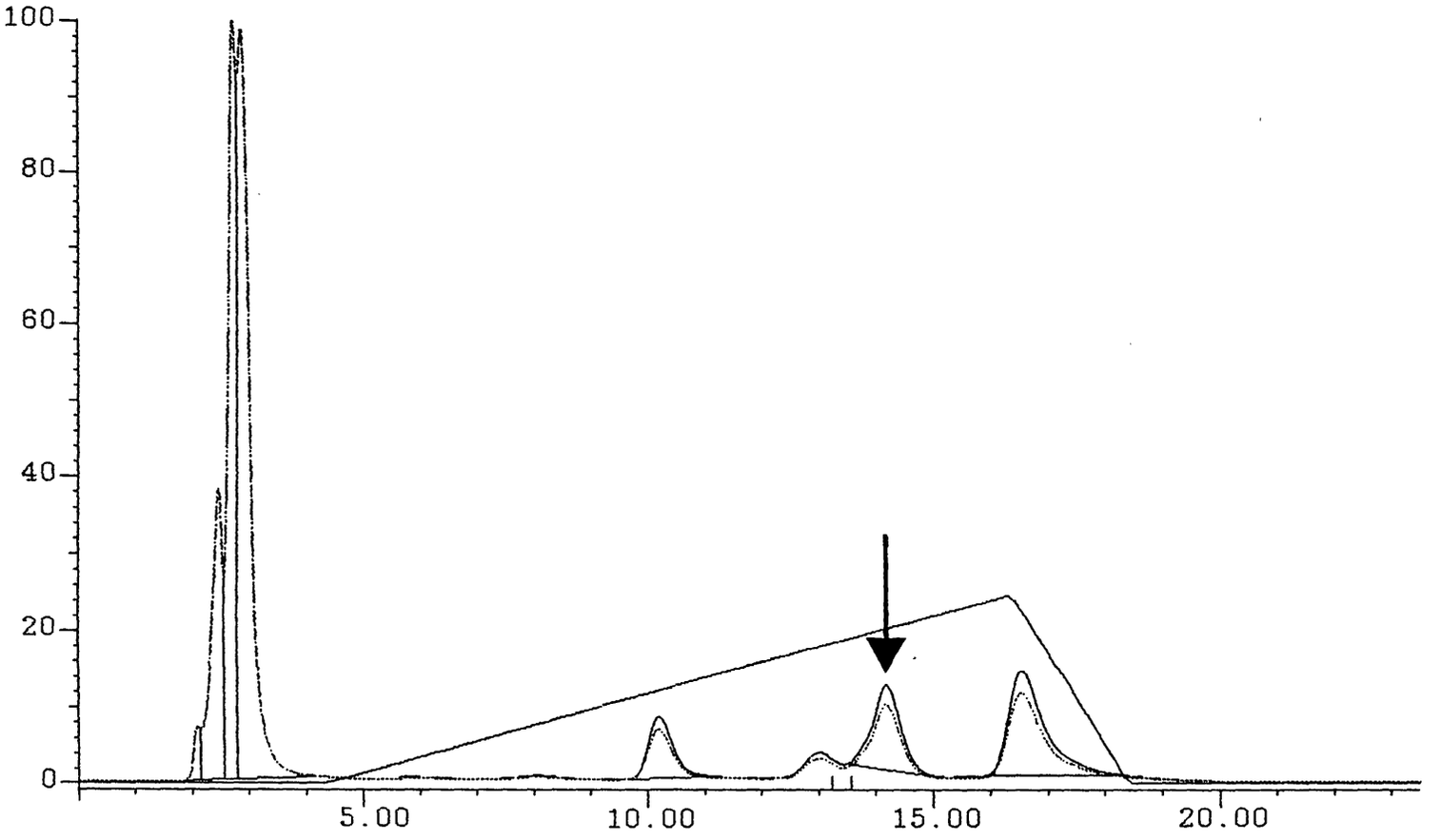


Figure 36. Chromatogram of PR1 (arrowed) from a culture filtrate of *M. anisopliae* (isolate ME1) fractionated on a strong cation exchange HPLC column. This is the yield of PR1 using the method of Patterson (1992). PR1 (arrowed) was eluted with a NaCl gradient.

proteinases other than chymoelastase were absent. In order to achieve this, PR2-like enzymes were removed from culture fluids by Q-sepharose chromatography and the eluent was freeze dried and subsequently fractionated on a S-sepharose column. Figure 37 shows the elution profiles on S-sepharose for the PR1 enzyme from 3 representative isolates of *Metarhizium* spp. The elution point of the individual enzymes differs and no two elution peaks were exactly alike (see appendix 4) reflecting differences in the pIs of the enzymes. As each isolate produced different quantities of PR1, it was essential to quantify the activity of each fractionation. Each peak of PR1 activity was collected, reduced in volume to 1 ml and the activity present determined using the standard assay. Furthermore, each sample was tested for PR2 and PR1 in the presence of a specific inhibitor (TEWI). In all cases no PR2 activity was found and all activities against Succ-Ala-Ala-Pro-Phe-pNA were inhibitable by TEWI. This suggests that the only endoproteinase activity present in the samples was PR1.

4.3.1.4 The hydrolysis of insect cuticle by PR1

The PR1 enzymes isolated from each isolate were then utilised to hydrolyse different insect cuticle types. Four types of cuticle were used for this study. (1) [^{3}H]-pupal cuticle from the lepidopteran *M. sexta*, the proteins of which are rich in glycine and appear to lack the Ala-Ala-Pro-Ala/Val (AAPA/V) repeat. (2) abdominal cuticle from pre-ecdysial adult *S. gregaria* abdominal (pharate) cuticle in which all proteins thus far sequenced contain the AAPA/V repeat motif. (3) abdominal cuticle from mature adult *S. gregaria*, which comprises, in part, pharate (pre-ecdysial) cuticle which has become sclerotised and is not readily available for hydrolysis. Thus, the AAPA/V motifs, whilst present, are not easily

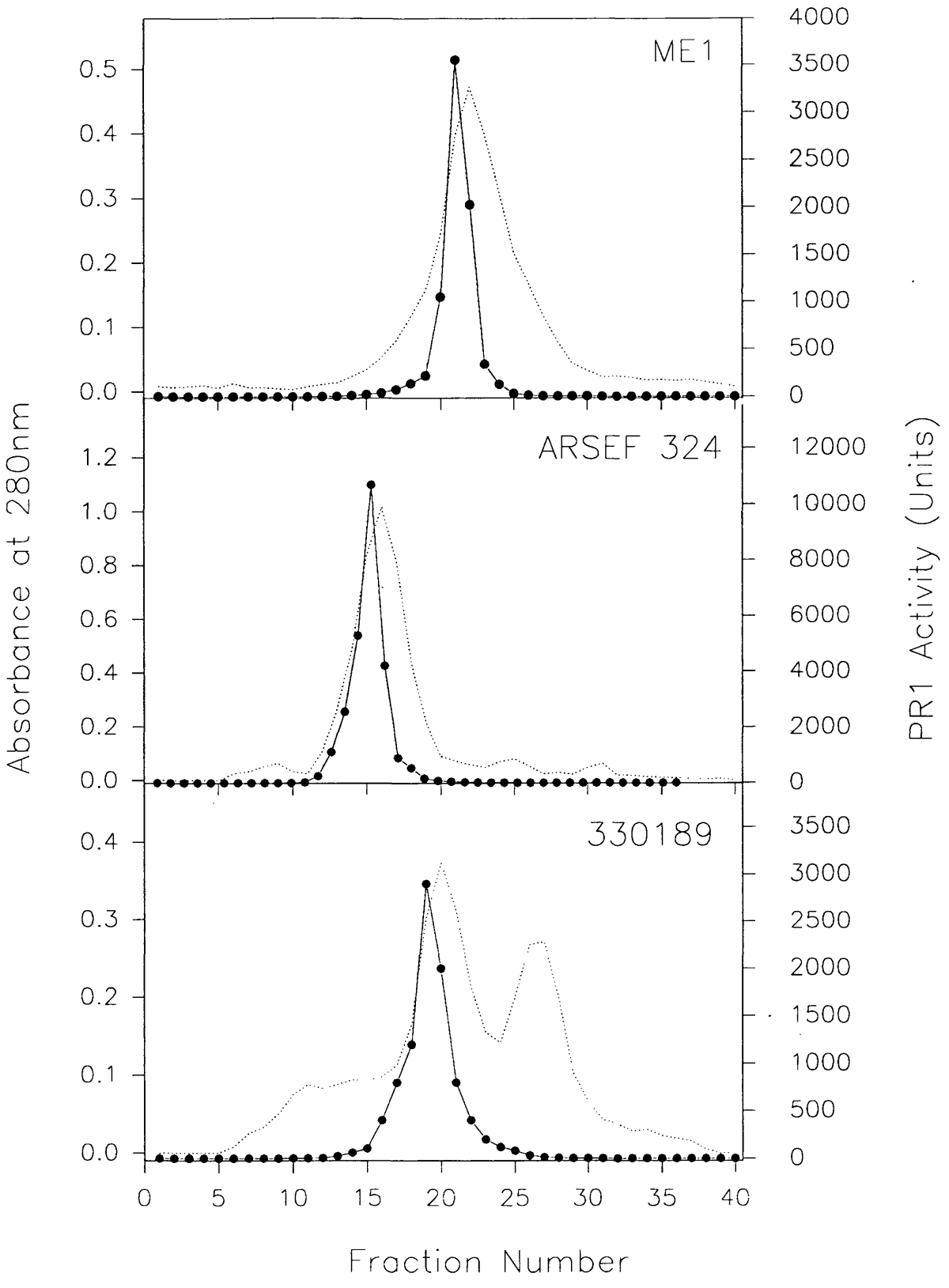


Figure 37. The elution profiles of PR1 enzyme from 3 isolates of *Metarhizium* spp. The enzyme activity was fractionated on an S-sepharose column
(•) PR1 activity; ( ) Absorbance at 280 nm.

accessable for enzymic action. Proteins in the post-ecdysial non-sclerotised part of the abdominal cuticle do not appear to have the AAPA/V repeat. (4) wing cuticle from mature adult *S. gregaria*, which contain pre-ecdysial cuticle proteins with lots of the AAPA/V sequence. Post-ecdysial cuticular protein in the wing is reduced compared to the abdomen and is of unknown composition. These 4 types of cuticle were selected because they represent cuticle with different protein compositions, sequence characteristics and degrees of sclerotisation (see discussion, section 4.4). The AAPA/V repeat is of particular significance because Succinyl-Ala-Ala-Pro-Phe-pNa is a particularly good substrate for PR1 from *M. anisopliae* (St. Leger *et al.,* 1986a).

The results of the hydrolysis of these different cuticle types are shown in Tables 27-30.

Table 27 shows the hydrolysis data for wing cuticle. The enzymes that hydrolysed the cuticle best were from 330189 (a good pathogen of locusts) and ARSEF 727ii (a poor pathogen of locusts). These enzymes released over 0.3 μg of protein whilst the enzymes from other isolates, with the exception of ARSEF 438, I91676 and 298061 released between 0.01 and 0.09 μg. The extent of hydrolysis with respect to the amount of soluble nitrogen released was equally variable between isolates. Isolate 330189 released 0.210 μg of alanine positive material whereas 727ii only released 0.128 μg.

The enzymes hydrolysed considerably more protein from abdominal cuticle than from wing cuticle (table 28). Interestingly, ARSEF 727ii released 3.7 times more protein from abdomen than from wing cuticle. However, there were no significant differences between the quantities of ninhydrin positive material released from wing and abdominal cuticle.

Table 27. Protein and ninhydrin analysis of locust wing cuticle hydrolysis by PR1 protease produced by 19 isolates of two *Metarhizium* spp.

Strain	µg protein released per mg cuticle per Unit PR1[b]	µg Alanine equivalents per mg cuticle per Unit PR1[b]
330189[a]	0.353 ± 0.059	0.213 ± 0.034
ARSEF 324[a]	0.036 ± 0.009	0.026 ± 0.007
324673[a]	0.060 ± 0.021	0.031 ± 0.006
ARSEF 2023[a]	0.048 ± 0.013	0.065 ± 0.020
ARSEF 438	0.102 ± 0.007	0.050 ± 0.007
ARSEF 439	0.038 ± 0.003	0.037 ± 0.004
ARSEF 440	0.048 ± 0.011	0.020 ± 0.004
ARSEF 727ii	0.305 ± 0.082	0.128 ± 0.029
I90574	0.010 ± 0.001	0.018 ± 0.001
I91633	0.050 ± 0.008	0.083 ± 0.021
I91676	0.124 ± 0.021	0.038 ± 0.007
298059	0.049 ± 0.008	0.091 ± 0.028
298061	0.152 ± 0.020	0.095 ± 0.030
299981	0.027 ± 0.006	0.054 ± 0.024
299984	0.040 ± 0.010	0.032 ± 0.009
ME1	0.022 ± 0.003	0.010 ± 0.003
Nr48	0.070 ± 0.014	0.032 ± 0.005
152222	0.048 ± 0.012	0.019 ± 0.003
168777ii	0.086 ± 0.018	0.046 ± 0.005

[a] Indicates *M. flavoviride* species, the rest are *M. anisopliae*.
[b] n=6; mean presented ± S.E.

Table 28. Protein and ninhydrin analysis of locust abdominal cuticle PR1 protease produced by 19 isolates of two *Metarhizium* spp.

Strain	µg protein released per mg cuticle per Unit PR1[b]	µg Alanine equivalents per mg cuticle per Unit PR1[b]
330189[a]	0.527 ± 0.058	0.178 ± 0.029
ARSEF 324[a]	0.169 ± 0.009	0.064 ± 0.007
324673[a]	0.139 ± 0.013	0.039 ± 0.008
ARSEF 2023[a]	0.335 ± 0.015	0.110 ± 0.006
ARSEF 438	0.127 ± 0.036	0.094 ± 0.016
ARSEF 439	0.204 ± 0.011	0.039 ± 0.004
ARSEF 440	0.088 ± 0.007	0.030 ± 0.002
ARSEF 727ii	1.026 ± 0.050	0.367 ± 0.021
I90574	0.102 ± 0.011	0.035 ± 0.006
I91633	0.216 ± 0.009	0.032 ± 0.003
I91676	0.184 ± 0.012	0.058 ± 0.005
298059	0.049 ± 0.008	0.050 ± 0.007
298061	0.557 ± 0.026	0.082 ± 0.003
299981	0.318 ± 0.024	0.086 ± 0.010
299984	0.159 ± 0.014	0.042 ± 0.006
ME1	0.034 ± 0.007	0.027 ± 0.005
Nr48	0.174 ± 0.010	0.053 ± 0.002
152222	0.057 ± 0.001	0.023 ± 0.002
168777ii	0.260 ± 0.017	0.075 ± 0.001

[a] Indicates *M. flavoviride* species, the rest are *M. anisopliae*.
[b] n=6; mean presented ± S.E.

Table 29. Protein and ninhydrin analysis of pharate locust cuticle hydrolysis by PR1 protease produced by 19 isolates of two *Metarhizium* spp.

Strain	µg protein released per mg cuticle per Unit PR1[b]	µg Alanine equivalents per mg cuticle per Unit PR1[b]
330189[a]	0.647 ± 0.058	0.063 ± 0.002
ARSEF 324[a]	0.630 ± 0.140	0.107 ± 0.028
324673[a]	0.165 ± 0.036	0.065 ± 0.031
ARSEF 2023[a]	0.446 ± 0.132	0.076 ± 0.031
ARSEF 438	0.454 ± 0.031	0.070 ± 0.025
ARSEF 439	0.093 ± 0.002	0.012 ± 0.002
ARSEF 440	0.687 ± 0.085	0.202 ± 0.019
ARSEF 727ii	1.375 ± 0.477	0.373 ± 0.075
I90574	0.079 ± 0.027	0.011 ± 0.001
I91633	0.510 ± 0.090	0.092 ± 0.034
I91676	0.387 ± 0.030	0.109 ± 0.021
298059	0.215 ± 0.057	0.063 ± 0.016
298061	1.919 ± 0.575	0.496 ± 0.033
299981	0.329 ± 0.175	0.793 ± 0.253
299984	0.390 ± 0.039	0.120 ± 0.016
ME1	0.718 ± 0.086	0.185 ± 0.031
Nr48	0.762 ± 0.210	0.157 ± 0.017
152222	0.284 ± 0.128	0.081 ± 0.025
168777ii	1.209 ± 0.216	0.262 ± 0.005

[a] Indicates *M. flavoviride* species, the rest are *M. anisopliae*.
[b] n=6; mean presented ± S.E.

Table 30. Protein and ninhydrin analysis of *Manduca* pupal cuticle hydrolysis by PR1 protease produced by 19 isolates of two *Metarhizium* spp.

Strain	DPM released per mg cuticle per Unit PR1[b]	µg Alanine equivalents per mg cuticle per Unit PR1[b]
330189[a]	18.91 ± 2.10	0.242 ± 0.032
ARSEF 324[a]	8.02 ± 1.32	0.121 ± 0.022
324673[a]	7.44 ± 0.41	0.102 ± 0.006
ARSEF 2023[a]	16.49 ± 2.27	0.217 ± 0.022
ARSEF 438	4.89 ± 0.58	0.102 ± 0.012
ARSEF 439	4.24 ± 0.54	0.053 ± 0.004
ARSEF 440	4.20 ± 0.67	0.066 ± 0.008
ARSEF 727ii	7.83 ± 0.97	0.149 ± 0.020
I90574	2.51 ± 0.29	0.064 ± 0.003
I91633	3.91 ± 0.45	0.083 ± 0.021
I91676	13.87 ± 0.99	0.191 ± 0.009
298059	7.15 ± 0.88	0.095 ± 0.046
298061	15.37 ± 1.30	0.111 ± 0.014
299981	5.87 ± 0.70	0.070 ± 0.018
299984	4.30 ± 0.30	0.117 ± 0.011
ME1	7.34 ± 0.22	0.162 ± 0.020
Nr48	4.70 ± 0.79	0.065 ± 0.011
152222	3.53 ± 0.89	0.055 ± 0.014
168777ii	4.30 ± 0.63	0.056 ± 0.004

[a] Indicates *M. flavoviride* species, the rest are *M. anisopliae*.
[b] n=6; mean presented ± S.E.

More protein was solubilised from pharate adult cuticle by the enzymes than from the abdominal or wing cuticle (table 29). In most cases the difference was significant for either wing or abdominal cuticle.

If these data points were plotted against the corresponding median lethal times and a correlation made, it was found that for all possible permutations, there was no significant correlation (table 31; figure 38).

However, if the μg protein released from one cuticle type was plotted against the μg protein released from another, there was a significant correlation in each case (table 32). Of interest was the fact that if the same comparisons were made between the alanine equivalents released, the only significant correlation was between the wing and abdominal cuticles.

A comparison of the data from the hydrolysis of locust cuticle types with that for *Manduca* cuticle was made. There was a significant correlation between enzyme activities against *Manduca* and wing cuticle but not between *Manduca* and the other two types (data not shown). Furthermore, the alanine equivalents showed a significant correlation between *Manduca* and wing but not for the other two.

In the case of *M. sexta* (Table 30), each PR1 released [^{3}H] peptides from the cuticle to a different degree. The pathogenicity for these strains against this insect has only been determined for strains ME1, 330189 and Nr48 (Kershaw, 1993). In the case of the pathogenic ME1, the enzyme released 7.34 DPM per mg cuticle whilst the non-pathogenic strains released 18.91 and 4.70 DPM respectively.

Table 31. Table of correlation coefficients for the median lethal time data versus the cuticle hydrolysis data.

Correlation made	r value	*P* value	Significance
µg protein released			
wing vs. MLT	-0.047	*P*>0.1	not significant
abdominal vs. MLT	0.041	*P*>0.1	not significant
pharate abdominal vs. MLT	0.087	*P*>0.1	not significant
µg alanine equivalents released			
wing vs. MLT	-0.013	*P*>0.1	not significant
abdominal vs. MLT	0.055	*P*>0.1	not significant
pharate abdominal vs. MLT	0.123	*P*>0.1	not significant

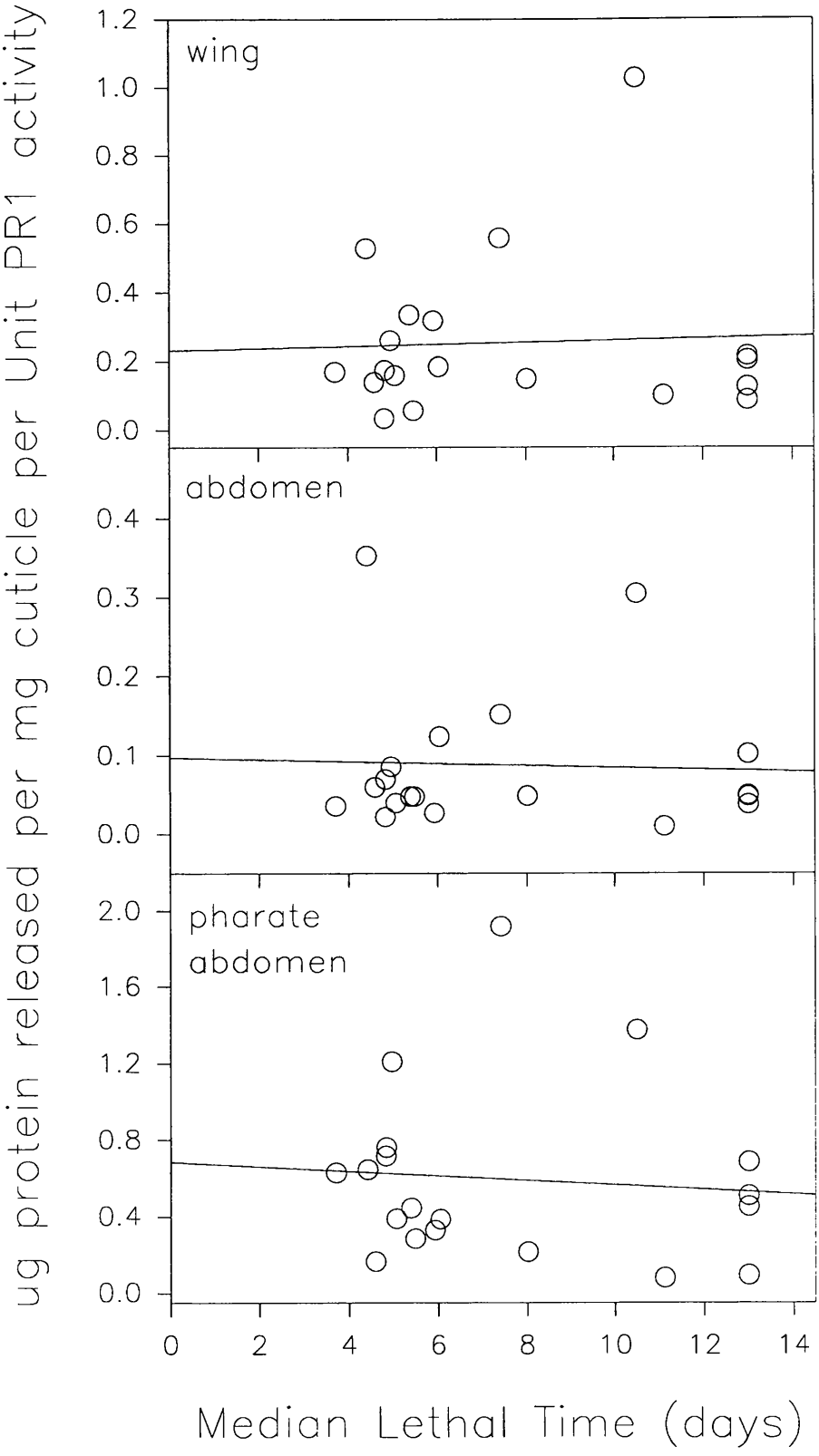


Figure 38: Correlations of the median lethal times for 19 isolates of *Metarhizium* spp. and the amount of protein released by PR1 hydrolysis of different cuticles

Table 32. Table of correlation coefficients for the data from cuticle hydrolysis.

Correlation made	r value	*P* value	Significance
μg protein released			
wing vs. abdominal	0.797	*P*<0.001	significant
wing vs. pharate abdominal	0.489	*P*=0.05-0.02	significant
abdominal vs.pharate abdominal	0.641	*P*=0.01-0.001	significant
μg alanine equivalents released			
wing vs. abdominal	0.664	*P*=0.01-0.001	significant
wing vs. pharate	0.290	*P*>0.1	not significant
abdominal vs.pharate abdominal	0.290	*P*>0.1	not significant

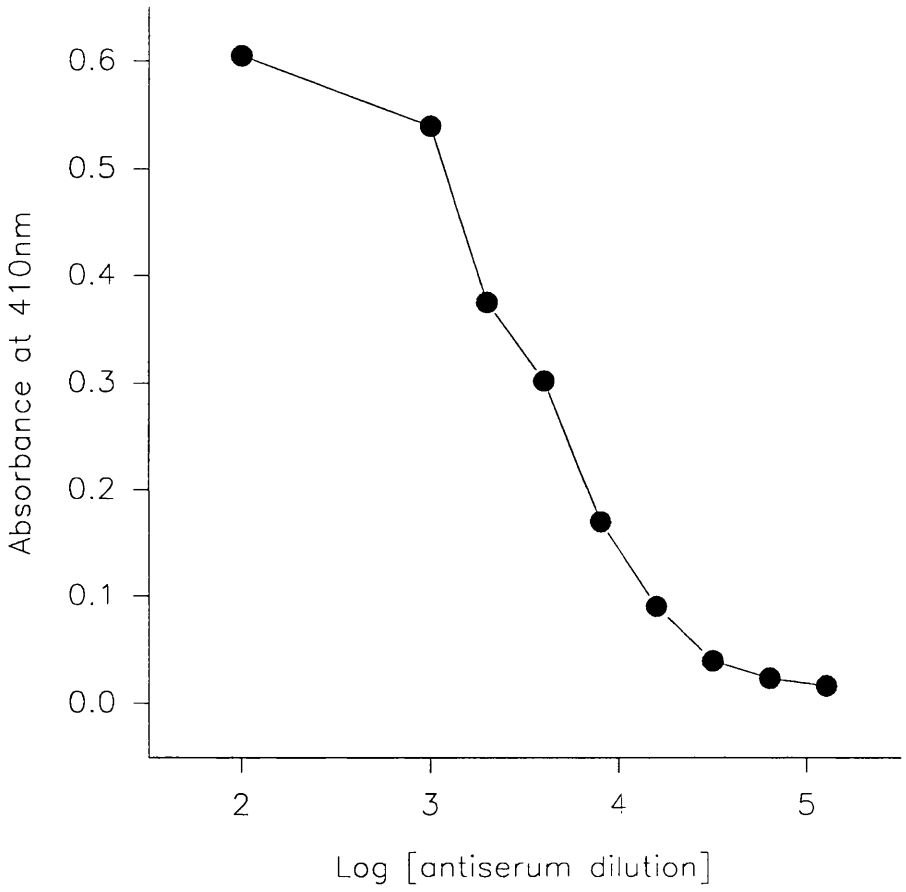


Figure 39. Antiserum dilution curve for antiserum raised against the PR1 protease purifed from *M. anisopliae* (isolate ME1)

4.3.2. Development of an ELISA to detect PR1 in insects during mycosis.

4.3.2.1. Production of an antiserum to PR1.

The antiserum prepared as described in the materials and methods showed a moderately high affinity towards PR1 and was capable of detecting 10 μg of PR1 at a dilution of 1/4000 (Figure 39). The antiserum was used at a working dilution of 1/400. At this concentration, the antiserum was able to detect as little as 0.125 μg of antigen (figure 40).

4.3.3.2. Specificity of anti-PR1 antiserum.

The specificity of the antiserum was tested. Competitive inhibition studies were performed with various enzymes competing for the antiserum in an incubation mixture containing native PR1 isolated from isolate ME1. The PR2-antiserum complex gave an absorbance 94% of the value for PR1 (the positive control; table 33). Proteinase K also bound the antiserum to a comparatively strong degree. Slight recognition (around 50% of the PR1 control) was observed with chymotrypsin, elastase and thermolysin. The other enzymes tested showed a low level of cross-reactivity.

The antiserum was also used to determine relatedness among PR1 enzymes isolated from 19 isolates. There was considerable variation in the ability of different PR1s to compete with PR1 from ME1 for recognition sites on the immunoglobulins in the antiserum (Table 34). For example, the antiserum recognised the PR1 from Nr48 with an absorbance reading which was 98% of that for ME1 (the positive control). Conversely, the antiserum only bound the PR1 from isolate I90574 with 10% of the efficiency of the ME1 PR1.

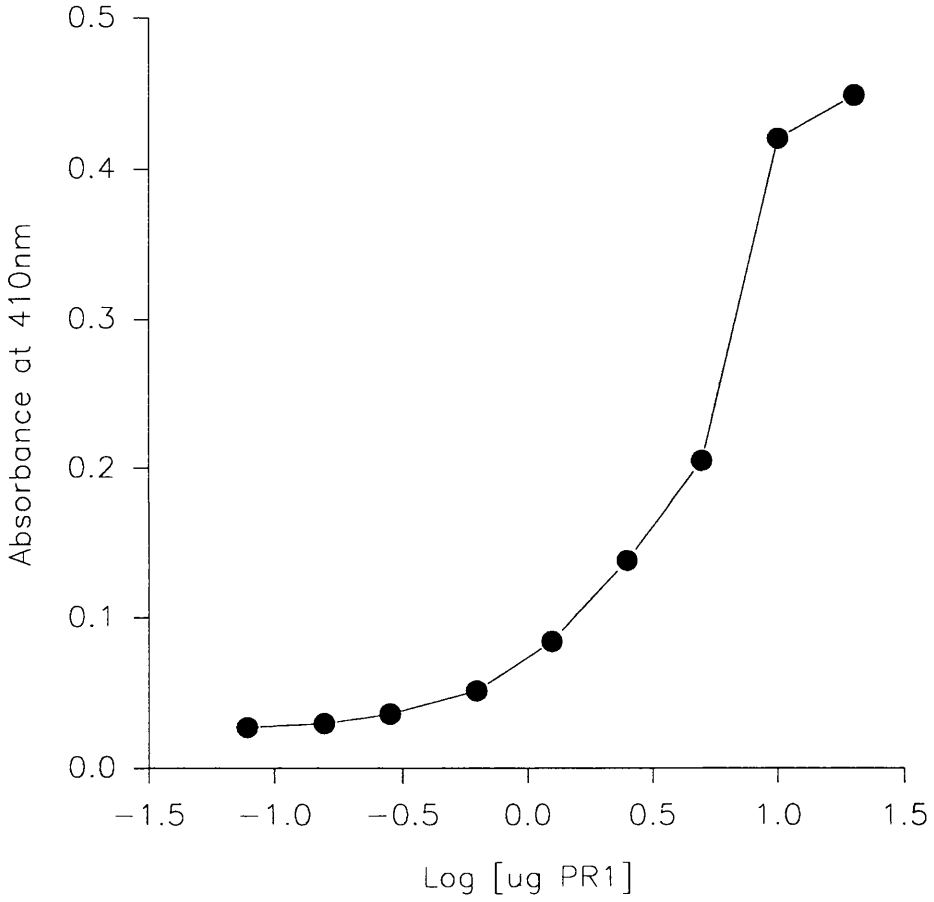


Figure 40. Analyte dilution curve for PR1 protease showing the detection limits of c 1/400 dilution of antiserum raised against PR1

Table 33. The cross-reactivity of anti-PR1 antiserum with various proteases.

Protease[a]	percent cross-reactivity[b]
PR1[c]	100.00
PR2[c]	93.39
Proteinase K	74.77
Chymotrypsin	54.65
Elastase	47.75
Thermolysin	46.55
Moulting Fluid protease (MFP-1)[d]	36.94
Papain	24.92
Subtilisin	23.72
Trypsin	23.72

[a] A range of proteases at a concentration of 0.1 mg/ml Tris-HCl buffer were used

[b] Results expressed as a percentage of the binding of the positive control which was PR1 isolated from *M. anisopliae* (isolate ME1) to which the antiserum was raised.

[c] PR1 and PR2 were isolated as described in section 3.3.3.3. and 3.3.3.2. respectively

[d] MFP-1 was kindly donated by Dr. R.I. Samuels

Table 34. The cross-reactivity of anti-PR1 antiserum with PR1 enzyme isolated from 19 different isolates of 2 *Metarhizium* spp.

Metarhizium Strain[a]	Percent cross-reactivity[b]
ME1[c]	100.00
330189[d]	43.44
324673[d]	76.38
ARSEF 324[d]	75.51
ARSEF 2023[d]	52.19
ARSEF 438[c]	34.40
ARSEF 439[c]	67.64
ARSEF 440[c]	67.64
ARSEF 727ii[c]	60.93
I90574[c]	10.00
I91633[c]	13.99
I91676[c]	80.76
298059[c]	75.51
298061[c]	94.46
299981[c]	60.93
299984[c]	74.05
Nr48[c]	98.25
152222[c]	86.59
168777ii[c]	95.04

[a] PR1 was isolated to monenzymic activity as described in section 3.3.3.3. All proteases were used at a concentration of 100 units activity/ml Tris-HCl buffer

[b] Results expressed as a percentage of the binding of the positive control which was PR1 isolated from *M. anisopliae* (strain ME1) to which the antiserum was raised.

[c] Indicates *M. anisopliae* isolate

[d] Indicates *M. flavoviride* isolate

Table 35. The fluorimetric detection of PR1 in the blood of *S. gregaria* infected with conidiospores of *M. anisopliae*.

Sample	Fluorimeter reading
Buffer alone	164 ± 1.76
PR1 and buffer	1934 ± 31.88
Locust plasma	124 ± 6.70
Locust plasma and PR1[a]	1708 ± 2.50
Locust plasma (Control)	124 ± 6.70
Locust plasma (Day 1)[b]	111 ± 6.51
Locust plasma (Day 2)[b]	115 ± 6.70
Locust plasma (Day 3)[b]	116 ± 6.54

[a] PR1 used at a final concentration of 0.025mg/ml

[b] Plasma taken from insects at different days post-inoculation. Insects were inoculated with 7.5 x 10^4 spores *M. anisopliae* (isolate ME1). S.E. shown; n=5.

Table 36. The fluorimetric detection of PR1 in the blood of *M. sexta* infected with conidiospores of *M. anisopliae* (isolate ME1).

Sample	Fluorimeter reading
Buffer alone	164 ± 1.76
PR1 and buffer	1934 ± 31.88
M sexta plasma	118 ± 2.63
M sexta plasma and PR1[a]	1739 ± 20.00
M sexta plasma (Control)	118 ± 2.63
M sexta plasma (Day 1)[b]	126 ± 14.03
M sexta plasma (Day 2)[b]	117 ± 2.98
M sexta plasma (Day 3)[b]	125 ± 2.84

[a] PR1 used at a final concentration of 0.025mg/ml

[b] Plasma taken from insects at different days post-inoculation. Insects were inoculated with 7.5 x 10^6 spores *M. anisopliae* (isolate ME1). S.E. shown; n=5.

Table 37. The ELISA detection of PR1 in the blood of *S. gregaria* infected with conidiospores of *M. anisopliae*.

Sample	% of maximal binding
PR1 alone[a]	100
Buffer alone	12.40 ± 0.97
Locust plasma (Control)	19.81 ± 1.04
Locust plasma (Day 1)[b]	17.00 ± 1.06
Locust plasma (Day 2)[b]	16.71 ± 0.77
Locust plasma (Day 3)[b]	17.32 ± 1.03
Locust plasma (Day 4)[b]	16.81 ± 1.04

[a] PR1 used at a final concentration of 0.025mg/ml
[b] Blood taken from insects at different days post-inoculation. S.E. shown; n=5.

Table 38. The ELISA detection of PR1 in the blood of *M. sexta* infected with conidiospores of *M. anisopliae*.

Sample	% of maximal binding
PR1 alone[a]	100
Buffer alone	12.40 ± 0.97
M. sexta plasma (Control)	20.68 ± 0.99
M. sexta plasma (Day 1)[b]	19.12 ± 0.96
M. sexta plasma (Day 2)[b]	18.67 ± 0.72
M. sexta plasma (Day 3)[b]	19.19 ± 1.13
M. sexta plasma (Day 4)[b]	17.74 ± 1.61

[a] PR1 used at a final concentration of 0.025mg/ml

[b] Plasma taken from insects at different days post-inoculation. Insects were inoculated with 7.5 x 10^6 spores *M. anisopliae* (isolate ME1). S.E. shown; n=5.

Furthermore, within the species, the binding was erratic. The antiserum recognised *M. flavoviride* 330189 with a reading 43.4% of that for ME1 whilst it recognised the enzyme from *M. flavoviride* 324673 with a 76% reading.

A comparison of the percentage binding of the enzymes when compared to that for ME1 enzyme against median lethal time revealed no significant correlation between the two sets of data.

4.3.3.3. Determination of PR1 in the blood of *M. sexta* and *S. gregaria* after infection by *M. anisopliae* (ME1).

4.3.3.3.1. Fluorimetry.

The fluorimetry assay for PR1 activity was found to be highly sensitive to the enzyme with a large deflection recorded for 100 units of activity (Table 35). Blood plasma from infected locusts and hornworms was diluted with an equal volume of buffer and assayed after 10 min. Results given in tables 35 and 36 show that there was no detectable enzyme activity in the blood of infected insects up to 3 days after infection. Moreover, there was no detection of enzymes in the blood of uninfected (control) insects.

4.3.3.3.1. ELISA.

It is possible that the lack of detection of enzyme activity by fluorimetry may be a result of inhibition of enzyme activity by endogenous protease inhibitors (see chapter 3). A competetive inhibition ELISA against infected blood showed that this was probably not the case as PR1 was not detected up to 4 days after infection in either *M. sexta* or *S. gregaria*. The antiserum did not cross-react with control blood either (Tables 37 and 38).

4.4. Discussion

The fungal penetration of insect cuticle is facilitated by a combination of mechanical force and enzymatic degradation (Charnley, 1984). Most fungi are not entomopathogenic because they are unable to penetrate insect cuticle. This may be because (1) they cannot overcome the host defences on the cuticle, (2) nutrient requirements (or other signals) for germination, appressorium formation and penetration are not met, (3) they do not have the necessary cuticle-degrading enzymes or (4) they cannot generate the mechanical force required to breach the cuticle. Fungi that are entomopathogenic such as *M. anisopliae, V. lecanii* and *B. bassiana* penetrate cuticle with the aid of a battery of extracellular cuticle degrading enzymes including protease, chitinases and lipases (Charnley and St. Leger 1991). Chymoelastases (PR1) and trypsin-like (PR2) proteases are a feature a number of a number of entomopathogenic fungi including *M. anisopliae, V. lecanii, B. bassiana* and *Nomuraea rileyi* (St. Leger *et al.*, 1987b) implying an important functional role for them in pathogenesis. The fungal cuticle degrading proteases have been shown to be determinants of pathogenicity in *M. anisopliae* (St. Leger *et al.*, 1988b) but, to date, their role in virulence has not been investigated in detail.. However, Hajek and St. Leger (1994) stated that many pathogen enzymes are important determinants of virulence because they enable the pathogen to coexist with the changing metabolic processes associated with the host's diseased state. Among pathogens, specificity and variation in virulence between isolates, at least at the level of the cuticle, may also relate to the parameters described above for non-entomopathogens. To which may also be added that the properties of the cuticle-degrading enzymes of the isolate concerned may be sub-optimal for the particular host cuticle. The present study shows variation between isolates in the amount of proteolytic

enzyme produced during growth *in vitro* on cuticle. The original inoculum for each isolate was the same and visual inspection suggested no large variations in the extent of growth between isolates. A comparison of maximal enzyme production and median lethal time indicated that there was no significant correlation between the two sets of data. However, enzyme activity was not expressed in terms of fungal dry weight. This was not possible because an insoluble source of nutrients (i.e. cuticle) was used. Failure to take into account variation in fungal biomass between isolates is an important omission. To circumvent this, ergosterol, a steroid peculiar to fungi, could be used as a marker molecule. Patterson (1992) found that ergosterol content of a fungus can be linked directly to its biomass. Furthermore, the DNA content of the fungus can also be used to relate growth to enzyme production.

Kershaw (1993) also found significant differences between isolates in PR1 and PR2 production during *in vitro* growth on cuticle. Both in the present work and in Kershaw (1993), PR2 usually appeared in culture 24h before PR1. This is consistent with a role for PR2 in the production of peptide inducers of PR1. PR2 (from ME1) has considerably less cuticle degrading ability than PR1. However, the products of limited locust cuticle digestion induce PR1 *in vitro*.

Quantitative differences in protease production *in vivo* between isolates could still influence virulence. Gupta *et al* (1991) observed minor differences in proteases produced by 4 isolates of *M. anisopliae* on two different types of cuticle. James (Unpubl.) found differences in PR1, PR2 and PR4 activity in extracts from locust wings infected with the isolates used in this study. However, she did not find any correlations between enzyme activity and MLT. In the present study, no significant correlation could be made between the MLT and the amount of PR1 or PR2 produced.

The PR1 enzymes purified from culture filtrates of the different isolates of *M. anisopliae* apparently differed in charge since they eluted in different fractions from the S-sepharose column. St. Leger *et al.*, (1994a) have shown in work published subsequent to this study, that there are 4 isozymes of PR1, with different pIs, in culture filtrates of a single isolate of *M. anisopliae*. It is probable that the PR1 preparations used in this study contained all possible isoforms of PR1 for each isolate used. In addition, since the isolates were grown on locust cuticle, the isoforms will have been produced in the "correct" proportion, bearing in mind possible differences in regulation between protease genes. Differences in charge between different PR1s will affect cuticle degrading ability since St. Leger *et al.* (1986c) have shown that electrostatic binding of PR1is a prerequisite for cuticle hydrolysis. The interaction of the PR1s with cuticle proteins, which will also vary in charge, may influence cuticle degradation within species (different body parts) and between species (Bidochka and Khachatourians, 1994a).

The data for the cuticle hydrolysis presented in this study showed a significant difference in the ability of the PR1 enzymes to digest different types of cuticle. Furthermore, the data suggest a hierarchy for the hydrolysis in the order pharate abdominal>abdominal>wing. These differences may be due to combination of physico-chemical properties of the cuticle. As mentioned above, the charge on the cuticle may have a significant outcome on the ability to bind PR1 protease. Furthermore, the amino acid sequences of the cuticular proteins from the different regions of the locust may have an effect. Approximately 100 distinct proteins have been isolated from the unhardened pharate cuticle of adult migratory locusts (Andersen *et al.*, 1986). Andersen *et al.*, (1993) have obtained the amino acid sequence of the locust protein Lm76 from total pharate exocuticle. It shared many of the characteristics of the proteins previously

isolated from the locust (Hojrup *et al.*, 1986a, b). The protein had a high content of alanine with a central hydrophilic region surrounded by 2 hydrophobic regions with pronounced internal homology due to the presence of seven repeats of the AAPA/V motif. This sequence of amino acids is very important as it is particularly good substrate motif for PR1 enzyme. The hydrophobic regions of all proteins sequenced thus far are dominated by repeats of this motif. Extremes are seen for the two wing cuticle proteins Lm70 and LmW7b which almost exclusively consist of this type of region (Krogh *et al.,* 1995). It is interesting to note that two proteins from locust endocuticle have been sequenced (Talbo *et al.*, 1990, Andersen *et al.*, 1993) which consist of almost exclusively hydrophilic regions. There are short hydrophobic regions but these do not contain the AAPA/V repeats.

In general, the sequences of proteins from the exocuticular proteins of locusts contain a distinctive repeat sequence of the hydrophobic motif AAPA/V often preceded by tyrosine. These regions were observed in all exocuticular proteins sequenced thus far (Andersen *et al.*, 1993). Extremes have been observed in the wing cuticle proteins Lm-70 and Lm-W7b which were found to be almost exclusively made up of this repeat. Jespersen *et al.* (1994) elucidated the primary structure of the Abd-5 endocuticular protein from *S. gregaria* and found it to be hydrophilic and lacking the AAPA/V sequence.

Most of the sequenced locust cuticular proteins have come from *L. migratoria.* Andersen (1988) compared the cuticular protein components of this insect with *S. gregaria.* The similarities in protein composition observed in the cuticles of these insects were so pronounced that the author proposed that all the main features of the structures of the proteins have been conserved. Thus it can be safely assumed that all previous results obtained from the cuticles of *L. migratoria* hold true for *S. gregaria* as

well. However, individual proteins have not been purified from *S. gregaria* and, accordingly, amino acid compositions and sequences have not been determined. All available evidence indicates that the differences between the adult pharate proteins of the two species are minor and that the main features have been conserved in the proteins (Andersen, 1988). However, differences between wing and femur cuticle in *L. migratoria* were also observed in *S. gregaria* indicating that the differences are functionally important.

Finally, the extent of sclerotisation of the host cuticle will play a role in the different degradative abilities of the PR1 enzymes from various isolates. Sclerotisation is defined as being the enzyme-catalysed incorporation of low molecular weight phenolic material into the cuticular structure, which leads to an increase in stiffness and resistance to degradation and digestion (Andersen, 1991). Both wing and abdominal cuticle was taken from sexually mature adult locusts. A large proportion of both these cuticles is sclerotised. However, the cuticle removed from pharate adults was not sclerotised as it had been removed prior to ecdysis. This suggests that sclerotisation is important in the efficiency of hydrolysis of cuticular proteins. As the pharate cuticle is not sclerotised, it is more susceptible to proteolytic activity. Bidochka and Khachatourians (1994b) have shown variations in the susceptibility of types of cuticle to proteolysis by culture filtrates of *Metarhizium s*pp. They showed that hindwing cuticle from grasshoppers lost 83% of its dry weight after treatment. This was in contrast to the 50% loss in dry weight for abdominal cuticle. However, only 8.3 μg protein was released from the hindwing whilst 44.2 μg were released from abdomen. The data presented here compares well with the findings in grasshoppers. However, in that study, culture filtrates rather than purified enzymes were used.

In the present work, the ability and, more significantly, the extent to which PR1s from different isolates can degrade cuticle was not correlated with virulence. However, a correlation was found between the ability of enzymes to hydrolyse different cuticle types. That is, despite differences between cuticle types in protein composition and sequence and extent of sclerotisation, an enzyme that hydrolysed well cuticle of one type was efficient also against other cuticle types. This may indicate that the charge of the enzyme (which may be the same for the 3 cuticles used) is of paramount importance in determining cuticle-degrading ability and that substrate specificity among PR1s is wide enough to accommodate the differences in protein sequences encountered in the 3 locust cuticular types used.

Since a good enzyme will degrade well cuticle from each source whilst a poor enzyme will be poor for all substrates, then one possible conclusion is that PR1 from a particular isolate may not increase the likelihood of penetration through one area of the cuticle over another. Correlation among isolates in ability to degrade different locust cuticle types was not apparent to the same extent between *Manduca* and locust cuticle. Since the majority of the isolates used (13/19) were originally isolated from Orthopterans, then perhaps PR1 activity relates to the host range of the isolate (specificity).

The kinetics of the hydrolysis of cuticle by PR1 are likely to be very complex because of the heterologous protein composition of the substrate. St. Leger *et al.* (1991) found that the shape and gradient of the hydrolysis vs time curve varied with enzyme concentration. There was a lag period which was ascribed to the cross-linked structure of the cuticle requiring substantial hydrolysis of peptide bonds before peptides were released into solution. Bidochka and Khachatourians (1994b) showed that a protease isolated from *B. bassiana* preferentially degraded high

molecular weight acidic proteins from the cuticle of *M. sanguinipes*. Subsequent work showed that protease adsorption was maximal in a broad pH range of 4-7 and was time dependent. The protease was shown to require carboxyl groups as well as an unsubstituted hydroxyl group with a carboxyl group for maximal adsorption.

In this study, an antiserum to the PR1 enzyme was developed that had a working dilution of 1/400. This was a good enough dilution to enable an ELISA to be developed. The sensitivity of the ELISA was also quite good with a lower detection limit of 0.125 μg PR1. Shimizu *et al.* (1993) have produced an antiserum to the protease produced by *B. bassiana* which can detect 31.6 ng of protein. The antibody produced here cross-reacted with a variety of PR1s extracted from different isolates of *Metarhizium* spp. The considerable extent to which the other proteolytic enzymes cross-reacted with the antiserum raised to PR1 from ME1 implies that either the cross-reacting enzymes share one or more epitopes or that the antiserum contains a number of different antibodies and thus lacks specificity. Alternatively, the differences could be due to different carbohydrate groups on the antigen. Apparent antigenic relatedness could not be correlated with MLT nor did it correlate to the geographical origins of the isolates. Segers *et al.*(1995) have raised an antiserum to PR1 from *M. anisopliae* (isolate ME1) which cross-reacts with a serine protease from the nematode pathogenic fungus, *Verticillium chlamydosporum*, but not with the PR1 from another strain of *M. anisopliae*. The antiserum also recognised the enzymes chymotrypsin, Proteinase K and PR2. This pattern is unexpected since the former has a primary structure homologous to PR1 whilst the latter is a trypsin-like enzyme with no sequence homology to PR1 (Smithson, Unpubl.). One explanation for this is that the PR1 preparation used to raise the antiserum was contaminated with PR2. This is unlikely as no PR2-like activity was detected in the enzyme preparations used.

Alternatively, the rabbit used to raise antisera had antibodies to a trypsin-like serine protease, for some reason, which cross-reacted with PR2. In contrast to these results, Segers *et al.* (1995) showed that a polyclonal antiserum against PRI from isolate ME1 cross-reacted with both elastase and Proteinase K but not chymotrypsin.

To date, PR1 has only been located in cuticle during host penetration (Goettel *et al.* 1989). PR1 is a broad spectrum protease, however, which could be useful to the fungus in the body cavity of the host either for nutrient acquisition or by cytotoxic activity against the host's cellular defences. The appearance of a protease in the blood of infected hosts has been shown by ELISA in *B. mori* infected with *B. bassiana* (Shimizu *et al.*, 1993). However, the authors did not indicate whether the antibody they had prepared cross-reacted with any endogenous proteases present in the host. In this study, the ELISA did not detect PR1 in the blood of either *M. sexta* or *S. gregaria* infected with *M. anisopliae* (isolate ME1). This may be due to the low sensitivity of the antiserum. However, a sensitive fluorimetric assay also failed to show any PR1 activity in the blood of mycosed insects. Alternatively, the nutrients available to the fungus may repress the production of PR1. Incidentally, PAGE analysis of proteins in the blood of locusts infected with *M. flavoviride* (isolate 330189), revealed only one protein band with a molecular weight in the same region as PR1 (28.6 kDa.) and this was in controls and decreased in quantity during infection (see chapter 2).

In summary, although there was strain variation in the amounts of protease produced *in vitro*, there was no significant correlation between enzyme production and virulence. No significant correlation was found between the amount of protein released from pharate abdominal, adult wing and adult pharate cuticle by PR1s and the MLT for each isolate. An

APPENDICES

APPENDIX 1

Anticoagulant Buffer (AC)

	Per litre
0.098 M NaOH	3.92
0.180 M NaCl	10.53
0.017 M EDTA (free acid)	6.32
0.041 M citric acid	8.60

440-450 mOs/Kg
pH4.5

Solution made up to 1 litre with endotoxin-free water (Camlab) and prepared using E-toxa cleaned grassware.

Basal salts media

	Per litre
KH_2PO_4	1.0g
$MgSO_4$	0.5g
Trace elements	1.0 ml
MES Buffer	10.65g (50 mM)

For the production of cuticle-degrading enzymes by *Metarhizium* spp., 1% (w/v) locust cuticle was added before autoclaving.

Czapek Dox liquid medium (modified).

Dissolve 33.4g of Czapek Dox and 5g of bacteriological peptone in 1 litre of distilled water. Mix well and adjust the pH to 6.5. Sterilise by autoclaving for 20 min at 10lb/sq. inch.

Hoyles Saline

	Per litre
0.234M NaCl	13.7g
0.006M KH_2PO_4	0.82g
0.004M $KHCO_3$	0.40 g
0.002M $CaCl_2$	0.22g
0.002M $MgCl_2$	0.19g

450 mOs/Kg; pH 4.4

Solution made up to 1 litre with endotoxin-free water (Camlab) and prepared using E-toxa cleaned grassware.

Locust Ringers saline (pH 6.8)

	per litre
140 mM NaCl	8.180g
10 mM KC	0.747g
4 mM $CaCl_2$	0.508g
4 mM $NaHCO_3$	0.336g
6mM NaH_2PO_4	0.936g
90 mM Glucose	16.210g

Sabarouds Dextrose Agar (SDA)

	1/4 Strength (w/v); Per Litre
1 % dextrose	10g
0.25 % mycological peptone	2.5g
0.5 % yeast extract	5g
2 % agar	20g

Mix components together over heat adjust pH to 5. autoclave at 15 psi for 15 min.

Buffers for SDS-PAGE

Separating Gel Buffer

1.5 M Tris-HCl	pH 8.8

Stacking Gel Buffer

0.75 M Tris-HCl	pH 6.8

Sample buffer

0.5M Tris-HCl (pH 6.8)	4.0 ml
Glycerol	1.0 ml
10% (w/v)	1.6 ml
0.05% (w/v) Bromophenol blue	0.2 ml

5 X Electrophoresis buffer

	per l
Tris base	15.0g
Glycine	72.0g
sodium dodecyl sulphate	5.0g

Make up with distilled water

Receipt for Separating gels

	10%	12%
Separating buffer	2.50 ml	4.10 ml
30% acrylamide	3.25 ml	2.50 ml
distilled water	4.20 ml	3.33 ml
10% ammonium sulphate	100µl	100µl
TEMED	10µl	10µl

Receipt for stacking gel

stacking gel buffer	750µl
acrylamide	125µl
distilled water	3000µl
ammonium sulphate	50µl
TEMED	5µl

Manduca Diet (Bell and Joachim, 1976).

Ingredients

Wheat germ	750g
Casein	350g
Sucrose	300g
Dry yeast	150g
Wesson's Salt mixture	100g
Sorbic Acid	15g
Cholesterol	10g
Methyl-*P*-hydrobenzoate	10g
Choline chloride	10g

This mixture was kept in an airtight plastic container at room temperature. A batch of diet consists of 504g of the above plus:

Ascorbic acid	12g
Aureomycin	0.3g
Vanderzant's vitamins	0.3g
10% formaldehyde	12ml
Raw Linseed Oil	6ml
Vegetable oil	6ml
Agar	45g

504g of the pre-mixture was put in a Waring blender and 1250ml of boiling distilled water was added. The agar was heated separately and transferred to a mixer when boiling. When the temperature of the mixture cooled to 70°C, the oils, formaldehyde, ascorbic acid, Vanderzant's vitamins and aureomycin were added. Mixture cooled in flow cabinet and stored at 4°C.

APPENDIX 2

Appendix 2. The effect of antiprotozoal drugs on the germination of *M. flavoviride* (isolate 330189)

% drug added to medium	% germination mean ± S.E.
0	19.90 ± 2.19
1	18.75 ± 1.17
2	18.66 ± 1.48
4	17.84 ± 0.43
5	16.80 ± 0.42
8	17.91 ± 2.13
10	16.50 ± 1.59
25	16.82 ± 0.59

n=4; 200 spores were counted per replicate. No significant differences among the data (analysis of variance; F=1.19; P=0.35)

APPENDIX 3.

Protocol for bioassay of fungal entomopathogens against *Schistocerca gregaria* and other grasshoppers to determine median lethal time (MLT).

Courtesy of C. Prior, IIBC, Silwood Park, Ascot, Berks, SL5 7TA.

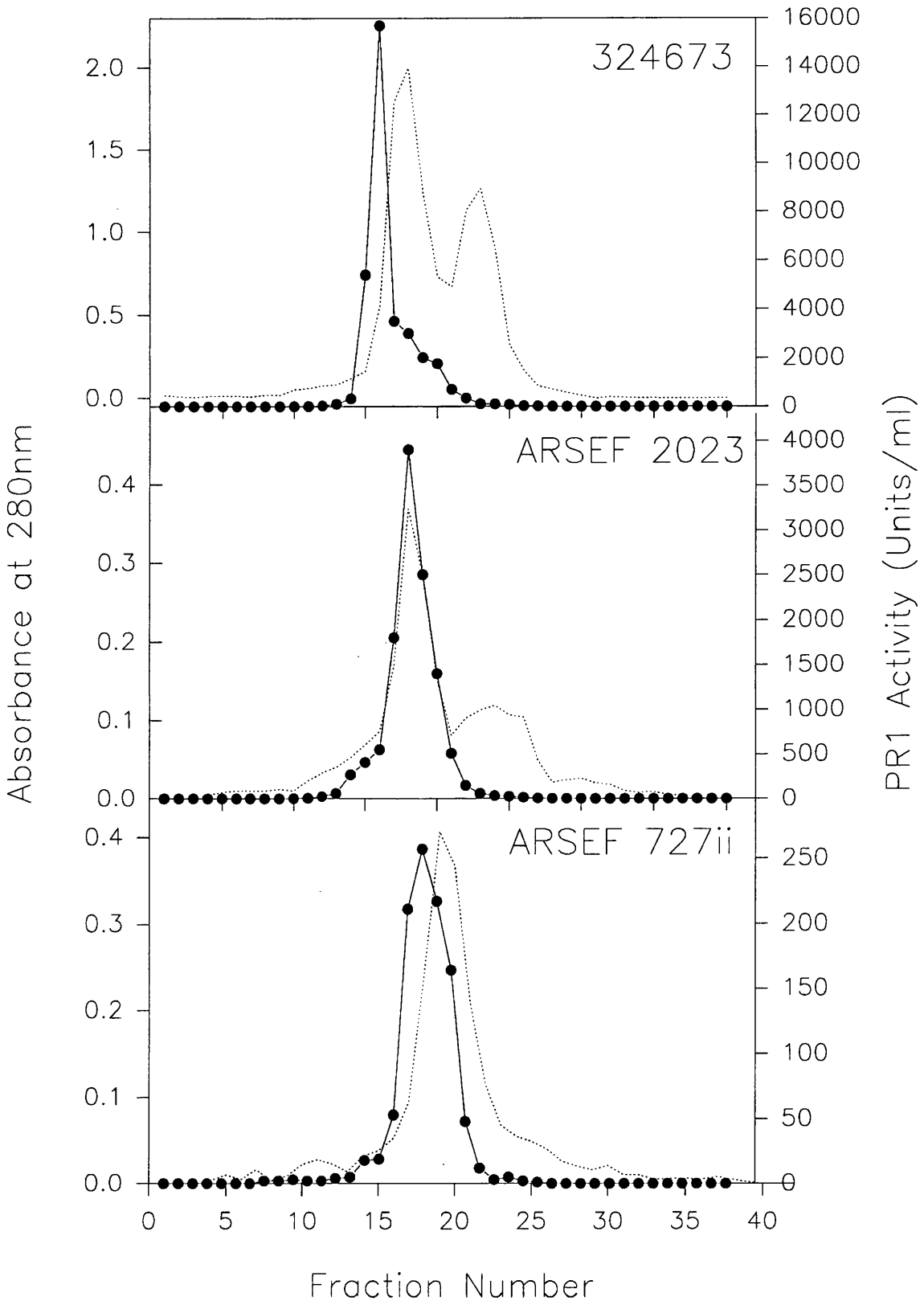
Adult locusts, 9-12 days post-fledgling were taken and placed in plastic food boxes with a piece of dry tissue paper in the bottom with no added food or water.

The spores of *Metarhizium* were grown on long agar slopes in 25ml Universal bottles containing 1/4 strength SDA (see Appendix 1). Cultures were incubated at 22-25°C between 10-30 days after inoculation. Conidia are harvested in cotton seed oil and adjusted to a final spore suspension of 3.75 x 107 conidia/ml.

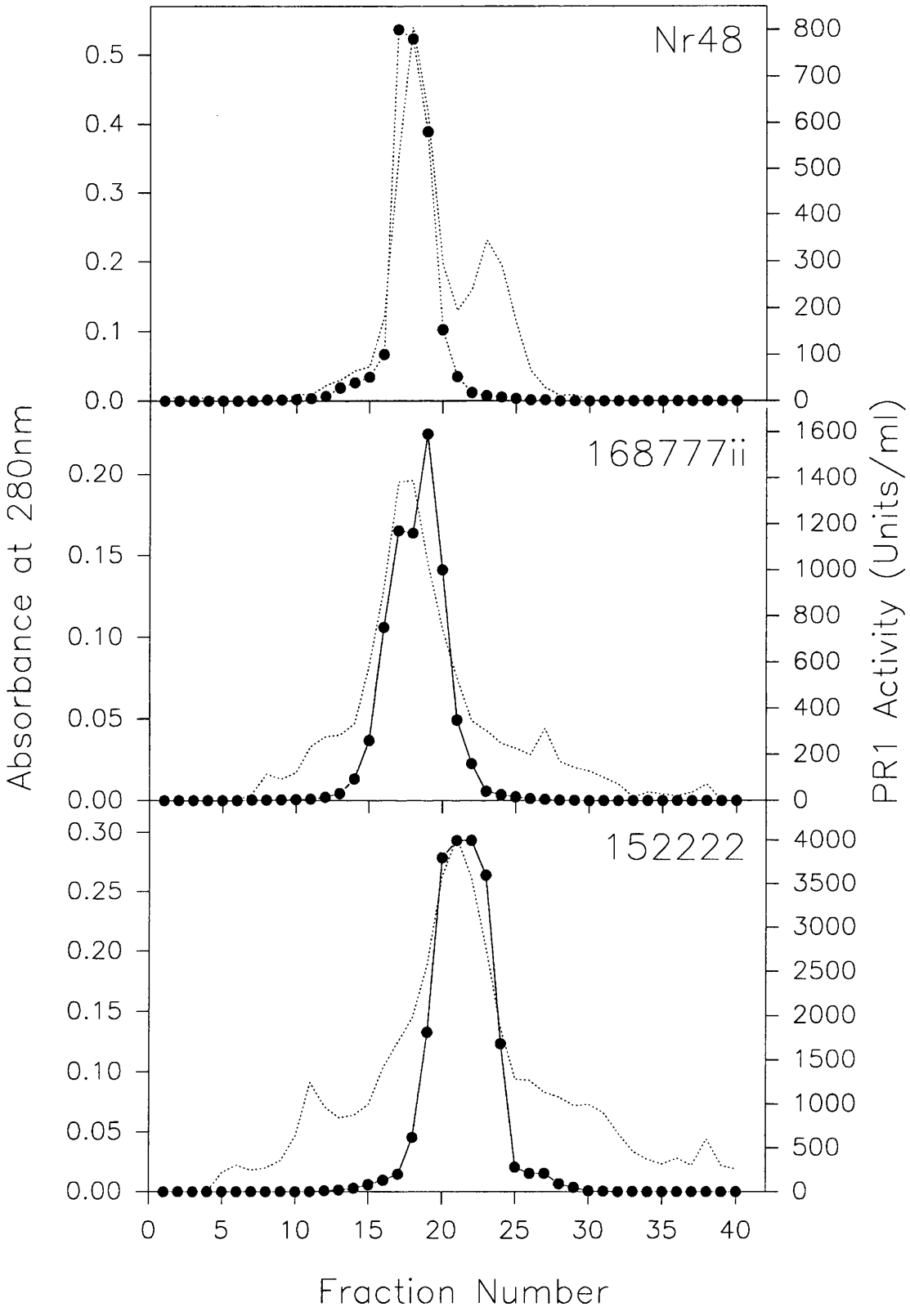
25 locusts were inoculated by placing 2µl of suspension behind the pronotum. The inoculated insect was replaced in the box and stored at 30°. Mortality was assessed daily. To determine whether death was due to the infection or other causes, the tissue paper in the box was saturated with water and the dead insect incubated for a few days in order to observe the outgrowth of the fungus on the outside of the cuticle. There is sometimes a rapid outgrowth of contaminating fungi (*Aspergillus* and *Mucor*) but the pathogen is most likely to be see on the antennae or on the tarsi.

Comparative data on time to death was analysed as estimates of median lethal time (MLT), which is the number of days to achieve 50% mortality.

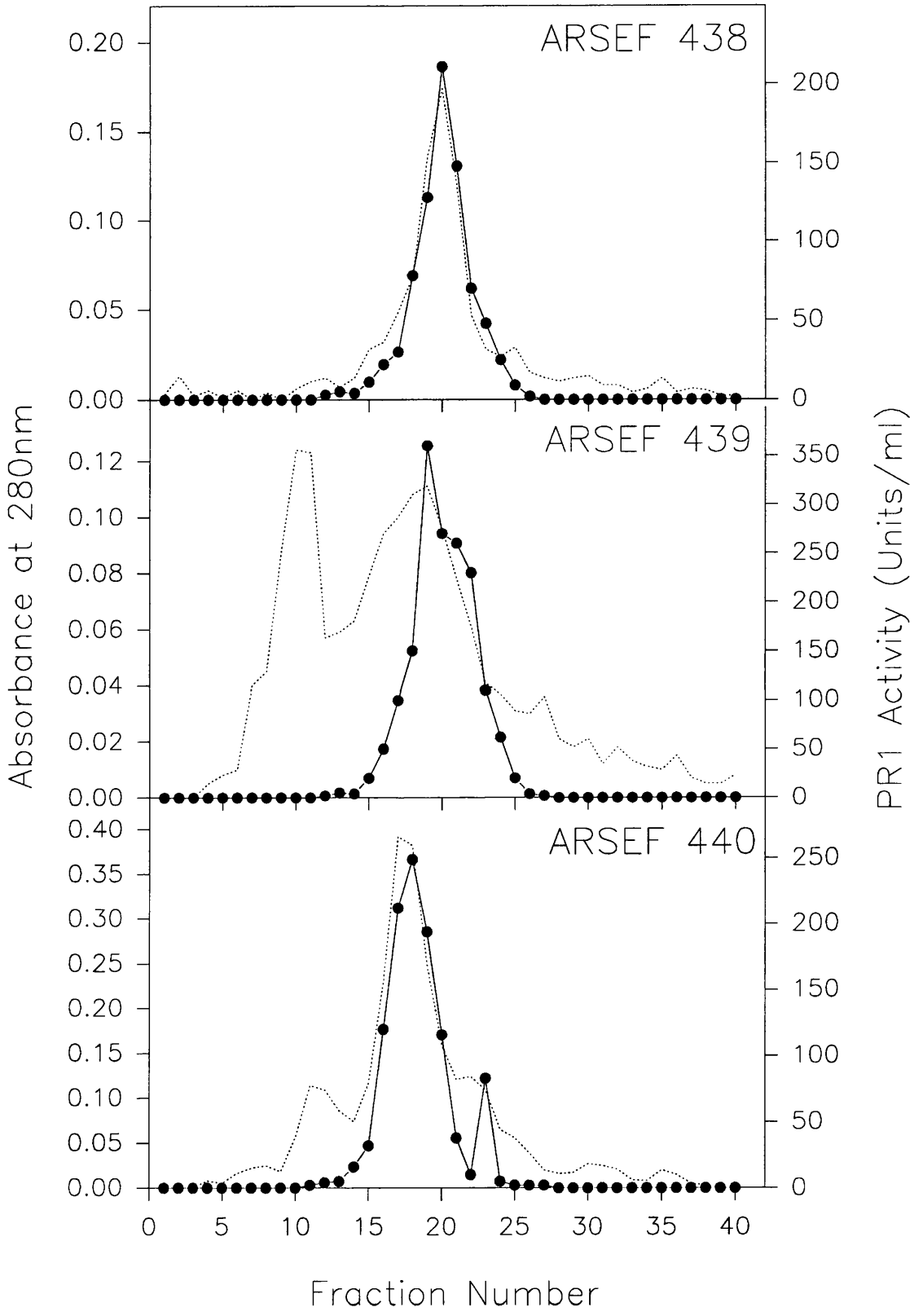
APPENDIX 4.
PR1 Elution Profiles



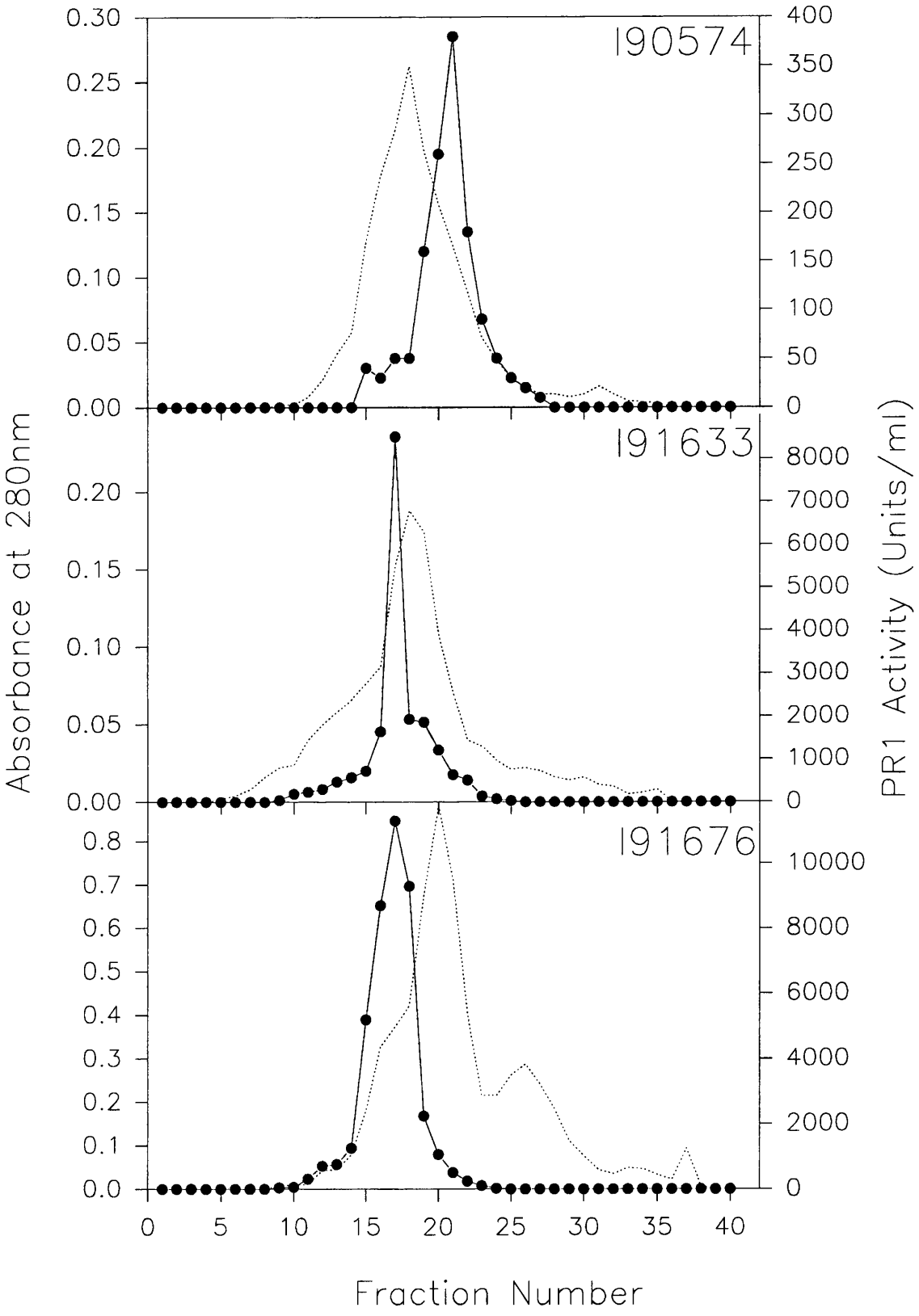
Appendix 3a. The elution profiles of PR1 enzyme from 3 isolates of *Metarhizium* spp. The enzyme activity was fractionated on an S−sepharose column
(•) Pr1 activity; (·····) Absorbance at 280 nm.



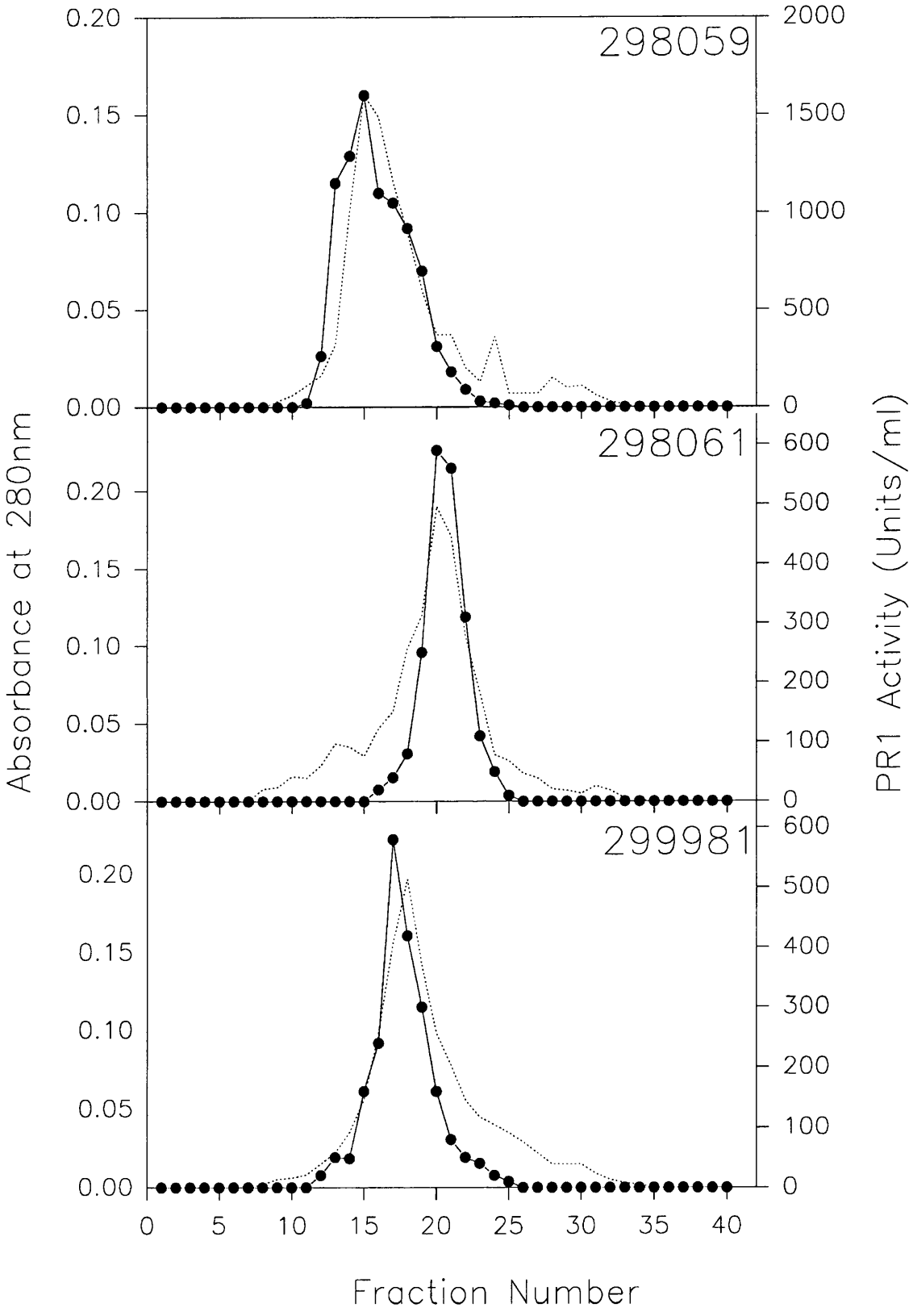
Appendix 3b. The elution profiles of PR1 enzyme from 3 isolates of *Metarhizium* spp. The enzyme activity was fractionated on an S−sepharose column (—●—) Pr1 activity; (——) Absorbance at 280nm.



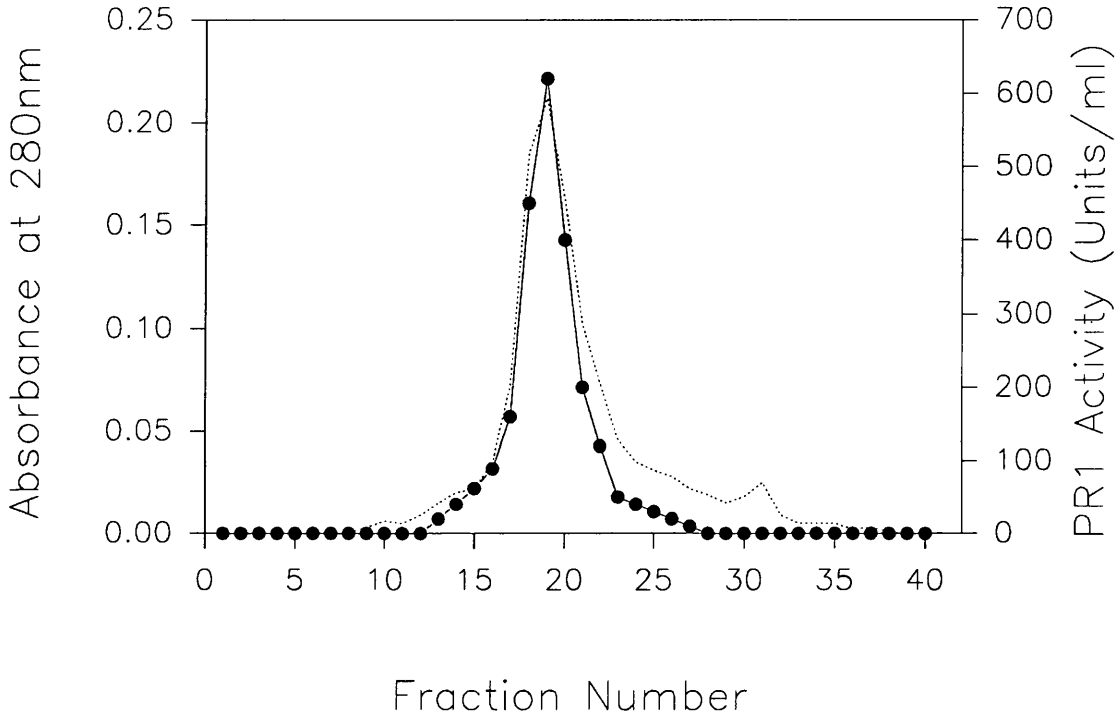
Appendix 3c. The elution profiles of Pr1 enzyme from 3 isolates of *Metarhizium* spp. The enzyme activity was fractionated on an S–sepharose column
(•) Pr1 activity; (·····) Absorbance at 280 nm.



Appendix 3d. The elution profiles of PR1 enzyme from 3 isolates of *Metarhizium* spp. The enzyme activity was fractionated on an S–sepharose column
(•) PR1 activity; (·····) Absorbance at 280 nm.



Appendix 3e. The elution profiles of PR1 enzyme from 3 isolates of *Metarhizium* spp. The enzyme activity was fractionated on an S–sepharose column
(•) PR1 activity; (·····) Absorbance at 280 nm.



Appendix 3f. The elution profiles of PR1 enzyme from an isolates of *Metarhizium* spp. The enzyme activity was fractionated on an S–sepharose column
(•) Pr1 activity; (·····) Absorbance at 280 nm.

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